Identification and characterisation of two haplosporidian parasites of oysters in north Western Australia.

Picture: An adult pearl oyster.

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A dissertation submitted to Murdoch University for the degree of Doctor of Philosophy.

Preface

The work described in this thesis is that of the author alone unless otherwise stated in the text. None of the work has been submitted for any other qualification at this or any other university.

Douglas Bearham

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Acknowledgments

While I'd like to claim all of this work as my own, it is not. I have benefitted from the ideas and advice of a large number of people and like all research it was built on the findings and discoveries of the researchers who came before. When it comes to naming names I would firstly, like to thank my supervisors Phil Nicholls and Shane Raidal for their hard work and invaluable advice and to Brian Jones for his knowledge and advice which was instrumental to the project. I'd also like to thank Zoe Spiers for her help throughout the course of the project. Zoe was involved in almost all aspects of the study in some way.

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- Bearham, D., Spiers, Z., Raidal,S., Jones.J.B.,Nicholls,P.K (2008) Spore ornamentation of *Minchinia occulta* n. sp. (Haplosporidia) in rock oysters *Saccostrea cucullata* (Born, 1778). *Parasitology*. **135**, (11) 1271-1280.
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List of Abbreviations, acronyms and definitions

bp	Base pair(s)
ddH_2O	Double distilled water
dNTP	dATP, dCTP, dGTP or dTTP
DIG	Digoxigenin
ECE	Epispore Cytoplasmic Extensions
EDTA	Ethylene diamine tetraacetic acid
H&E	Hematoxylin and Eosin
Inflammation	Infiltration of haemocytes in oyster tissues
ISH	In situ hybridization
Longline	Rope with anchors and bouys attached. Common method used to maintain pearl oysters in the water column.
MDS	Multidimensional scaling
min	Minutes
MSN	Minimum Spanning Network
NPV	Negative predictive value (or number of negative test results for truly disease-free animals divided by the total number of negative test results.
OIE	Office Internationale d'Epizootie or World Organisation for Animal Health.
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Pearl Oyster	Pinctada maxima
Rock Oyster	Saccostrea cucullata
s	Seconds
SEM	Scanning Electron Microscopy
Sensitivity	The number of disease-free animals that test negative divided by the number of truly disease-free animals.
SSC	Standard Saline Citrate
SSU	Small Subunit region of the rRNA gene
Spat	Juvenile pearl oysters
Sydney rock oyster	Saccostrea glomerata
TBSBT	Tris-buffered saline containing 3% bovine serum albumin and 0.1% Triton X-100
TE	Buffer containing 10 nM Tris – HCl (pH 8.0), 1 nM EDTA
TEM	Transmission Electron Microscopy
Tris	Trishydroxymethylaminomethane
Tropical oyster	Saccostrea echinata

Summary

A cryptic haplosporidian parasite was detected infecting rock oysters from the Montebello Islands in north-western Australia using a PCR targeting the parasite's small ribosomal subunit gene. The PCR products were cloned and sequenced along with the remaining sections of the parasite's SSU rRNA gene. Phylogenetic analysis of the sequence generated indicated a *Minchinia* species (Haplosporidia). The SSU sequence generated was used to develop two *in situ* hybridisation assays to visualise the parasite in H/E sections as well as a PCR assay to detect the parasite. The molecular assays were assessed for specificity and sensitivity and were then used to compare the parasite to previous haplosporidian parasite infections of pearl oysters. Both assays produced positive results from the infected pearl oysters but not from other closely related haplosporidian species. An SEM and TEM electron microscopy analysis was performed on spores from both parasite species. The spores of the pearl oyster parasite had two spore wall filaments wound around the spore originating for a posterior thickening while the spores of the rock oyster parasite were covered in microtubule-like structures. These data suggests pearl oysters where co-infected with both the *Haplosporidium* sp. and the *Minchinia* sp. detected in rock oysters. No evidence of a posterior thickening could be found on the spores of the rock oyster parasite. Attempts to detect the parasite at the previous geographic sites of its detection in pearl oysters resulted in detection of the *Minchinia* species in tropical oysters in the Kimberley region of Western Australia by *in-situ* hybridisation.

Thesis Flow Diagram

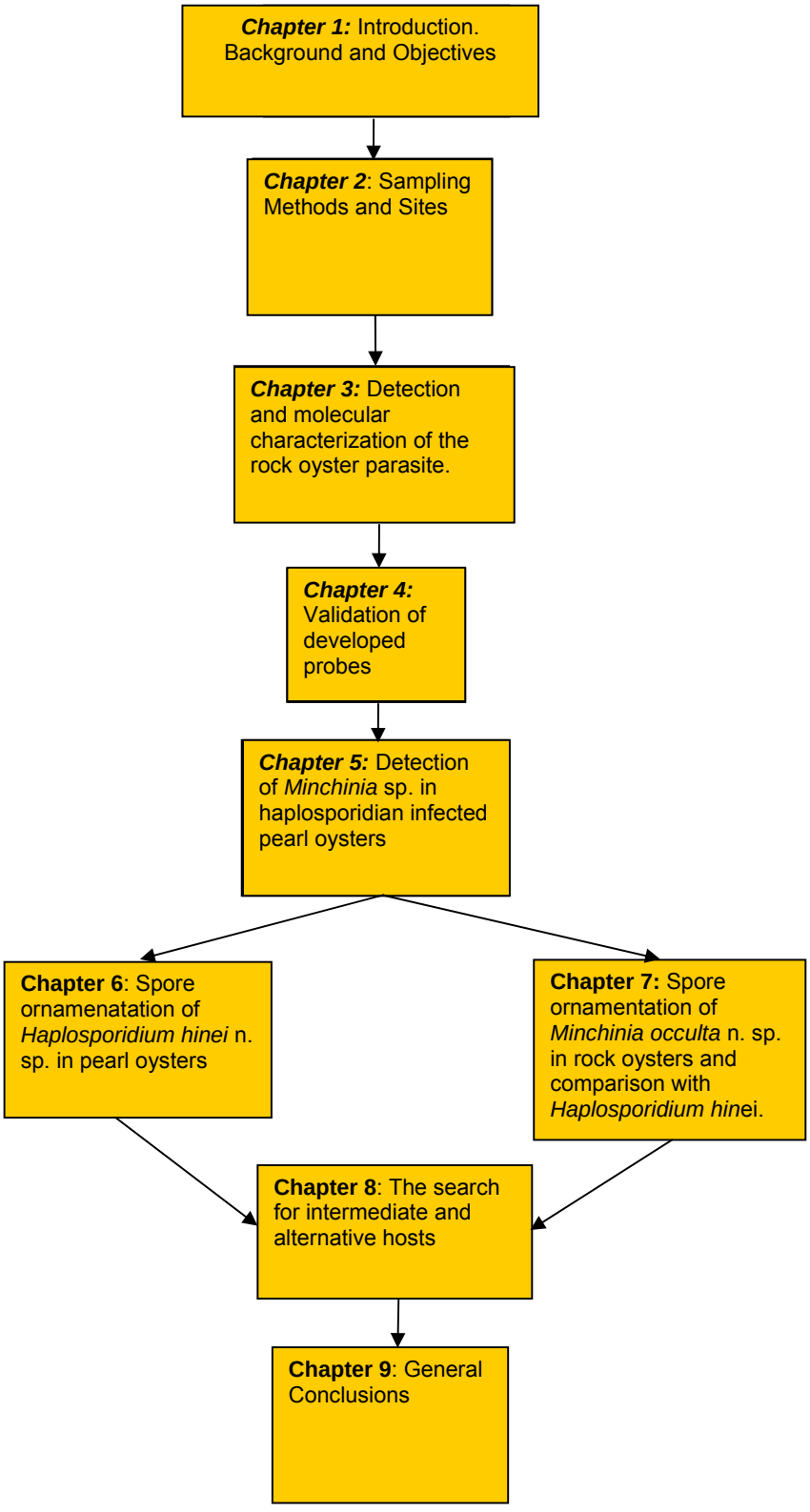


Table of Contents

List of Figures.

List of Tables

Chapter 1 Introduction.

1.1 Introduction to bivalve aquaculture.

Shellfish farming represents almost a quarter of world aquaculture output, and is still growing rapidly (Grizel, H., 2003). Its history has been characterised by periods of development and profitability, but also by crises. Most often, these crises are due to over-exploitation or diseases. The latter, when they occur have severely disrupted production, and can constitute a major hurdle to shellfish aquaculture (Grizel, H., 2003). Australia's most valuable shellfish culturing industry is the pearling industry (ABARE, 2007). The industry is second only to rock lobsters in export value (Figure 1.1)

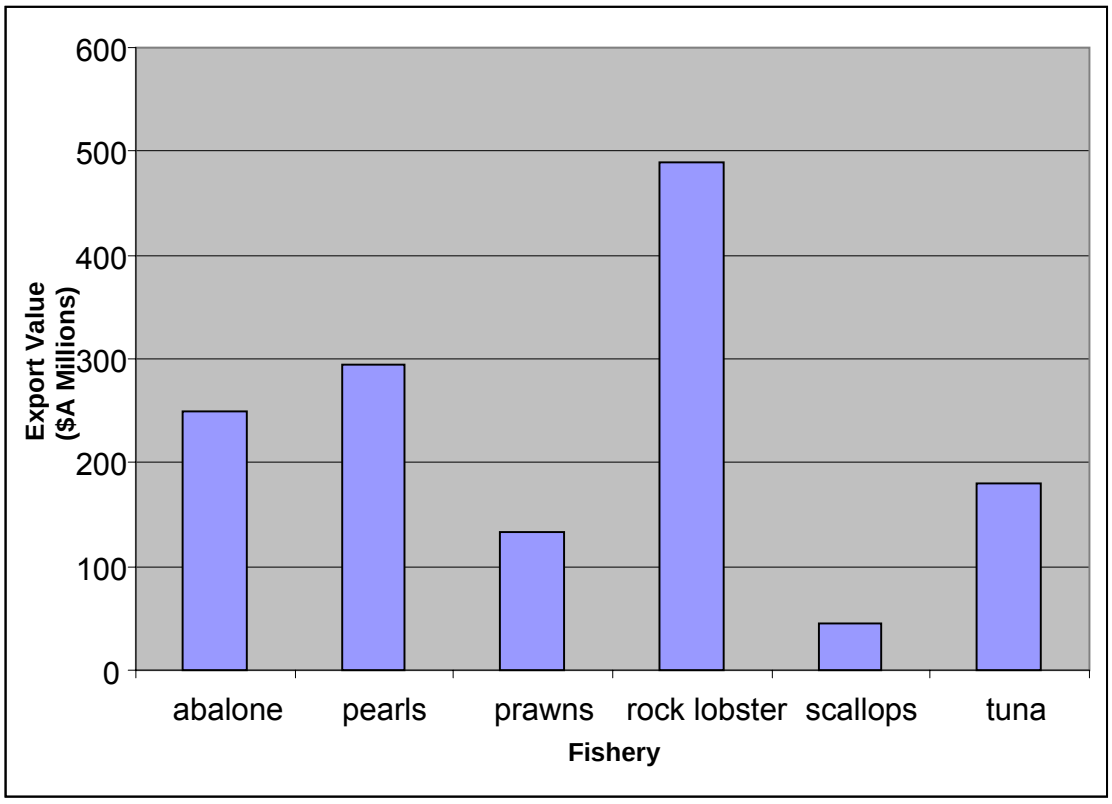


Figure 1.1 Total value of all Australian fisheries by export value ($A; ABARE, 2007).

1.2 The pearling industry.

The Australian pearl oyster industry is one of the world's largest producers of the highly prized, south sea pearls that come from the silver lipped pearl oyster *Pinctada maxima* (Jameson, 1901). The product is marketed primarily to Japan, the United States of America, Hong Kong and Europe (ABARE, 2007). The pearling industry based on *Pinctada maxima* extends from northern Australia to several Asian countries (including Indonesia, Malaysia and Thailand).

The pearling industry in Western Australia is vertically integrated and involves four basic activities (Fletcher, W. *et al.*, 2006):

- Collection of wild pearl oysters.
- Seeding oysters involving the implanting of nuclei.
- Grow out of seeded oysters.
- Marketing of the product.

The industry is primarily based on the collection of wild oysters that are seeded on the fishing grounds where they are dumped on the seabed (Fletcher, W., Friedman, K., Weir, V., McCrea, J. and Clark, R., 2006). The oysters are turned over approximately once per month to prevent rejection of the implanted nucleus and to aid in the formation of round pearls. The oysters are transported to farms at the end of the fishing season where they are usually cultured in panels suspended from long-lines. Pearls are harvested after approximately 2 years on the farm. Generally, about half of these harvested oysters are reseeded with a nucleus of a similar size to the cultured pearl that is removed depending on the health of the oysters. Since the oysters can

produce pearls several times over their lifespan, they are a valuable livestock and understanding mortality issues for this industry is therefore of paramount importance.

The international market price of pearls has declined by approximately 75% since 1997, primarily as a result of increased production by overseas competitors. The quota controlled Australian industry hopes to improve profitability by intensification through increased farm densities, and hatchery production of selectively bred juvenile pearl oysters. However, the survival of hatchery raised spat can be low after they are deployed to the open ocean and the causes of spat mortalities are not well described (Humphrey *et al.*, 1998).

As a result, production from spat is less predictable than for adult wild oysters. For this reason, disease management is likely to play a more important role in the industry than it has in the past.

1.3 Current disease management in the pearling industry.

Currently, disease does not have a major influence on the pearling industry in Western Australia (Anon, 2004). One possible explanation for this is that Western Australia is isolated relative to the major areas of shellfish disease concern worldwide (Anon, 2004). The transport of live animals for breeding or stocking has caused significant disease problems internationally (Burreson, E.M. and Ford, S.E., 2004). Previously, this movement could have been undertaken without regard for, or in spite of, the known disease risk. Disease management also plays an important role in ensuring the pearling industry remains relatively pathogen free.

The management of the industry in Western Australia is complex (Brayford, H.G. and Paust, G., 2000) and includes a diagnostic capacity to identify causative agents and the existence of passive and targeted surveillance programs aimed at identifying endemic diseases (Jones, J.B. and Creeper, J.H., 2006) to ensure that only the movement of healthy oysters occurs (Hine, P.M., 2000; Jones, J.B. and Creeper, J.H., 2006).

1.4 Major pathogens affecting Australian pearl oysters.

The most significant disease problem to have affected the Australian pearling industry to date is vibriosis, mainly due to the bacterium *Vibrio harveyi* (Dybdahl, R.E. and Pass, D.A., 1985). Mortalities occurred from about 1974 onwards and on occasion reached 90 percent, although 30 to 60 percent was more common (Dybdahl, R.E. and Pass, D.A., 1985). Investigations found that the mortality was related to transporting pearl oysters from fishing grounds to lease sites (Dybdahl, R.E. and Pass, D.A., 1985). During the longer fishing periods, collected oysters would remain in vessel transport tanks for up to four or five days. The oysters were kept in high densities with inadequate water circulation and higher than usual water temperatures. This caused a build-up of *Vibrio* in the transport tanks (Dybdahl, R.E. and Pass, D.A., 1985). Improved transport methods have all but eliminated the problem.

Recently, the industry has suffered serious mortalities from a condition named as oyster oedema disease (OOD). The mortalities were greatest in juvenile oysters but mature oysters have also been affected. Mortalities were most intense in and around Exmouth Gulf. An infectious agent has not yet been identified.

Haplosporidian parasites have also been detected in pearl oysters (see below).

1.5 Phylum Haplosporidia (Caullery & Mesnil, 1899).

The phylum Haplosporidia comprises a small group of spore forming obligate protozoan parasites of a number of fresh water and marine invertebrates (Burreson, E.M. and Ford, S.E., 2004). Haplosporidian parasites can be amongst the most dangerous of all molluscan pathogens particularly where naïve hosts are exposed to the parasite (Anon, 2002; Burreson, E.M. *et al.*, 2000b). At present there are 39 recognised species in the phylum, however numerous others have been reported, but not specifically identified, from many invertebrate hosts. They have received considerable attention since several are known to cause significant mortalities in commercially important mollusc species.

1.5.1 The characteristics of the Haplosporidia.

The Haplosporidia were most recently characterized morphologically as possessing ovoid, walled spores lacking polar filaments or polar tubes, and with an orifice at one pole. The orifice is covered either externally by a hinged lid or internally by a flap of wall material (Cavalier-Smith, T. and Chao, E.E.Y., 2003).

Currently, there are four genera allocated to the phylum; *Urosporidium* Caullery and Mesnil 1905; *Minchinia* Labbe, 1895; *Haplosporidium* Caullery and Mesnil 1899 and *Bonamia* Pictot, Comps, Tige, Grizel and Rabouin 1980 (Reece, K.S. *et al.*, 2004). The primary taxonomic feature used to differentiate species and genera within the Phylum is the morphology and origin of spore ornamentation, usually described as filaments, tails, wrappings or extensions.

Presently, the genera *Haplosporidium* and *Bonamia* are separated from the *Minchinia* based on the origin of the spore ornamentation, from either the spore wall in the case of the *Haplosporidium* and *Bonamia*, or from the epispore cytoplasm in the *Minchinia* (Azevedo, C., 2001; Burreson, E.M. and Ford, S.E., 2004; Burreson, E.M. and Reece, K.S., 2006; Carnegie, R.B. *et al.*, 2006; Hine, P.M. and Thorne, T., 1998). The spore ornamentation from only one species of *Bonamia* has been characterised. That species *Bonamia perspora* has been found to possess strap like projections derived from the spore wall (Carnegie, R.B., Burreson, E.M., Hine, P.M., Stokes, N.A., Audemard, C., Bishop, M.J. and Peterson, C.H., 2006).

Haplosporidian parasites have devastated production of some commercially important mollusc species. *Haplosporidium nelsoni* is the aetiological agent of the highly pathogenic Multinucleate Sphere X (MSX) disease in the eastern oyster *Crassostrea virginica.* The *Haplosporidium* was evidently introduced to the western seaboard of the United States by importation of infected Pacific oysters (*Crassostrea gigas*) from Japan (Burreson, E.M. *et al.*, 2000a). It also appeared on the eastern seaboard of the United States, where it has had devastating effects on wild oyster populations and hindered oyster aquaculture development in the region (Ford, S.E. and Haskin, H.H., 1982). *Haplosporidium nelsoni* has also been found to occur in France, probably the result of the importation of infected oysters (Renault *et al*, 2000).

Haplosporidium costale, the agent of Sea Side Organism (SSO) disease, is morphologically similar to *H. nelsoni* and has also been responsible for mortalities of eastern oysters on the US mid-Atlantic coast (Burreson, E.M. and Reece, K.S., 2006).

Bonamia ostrea, pathogenic in the European flat oyster *Ostrea edulis*, has devastated the flat oyster industry in Western Europe. A microcell parasite that closely resembled *B. ostreae* in *O. edulis* has been reported from California. (Elston, R.A. *et al.*, 1986). The presence of *B. ostreae* in France was later traced to the importation of oysters from California (Elston, R.A., Farley, C.A. and Kent, M.L., 1986). More recently, this parasite was also found on the east coast of the United States (Friedman, C.S. and Perkins, F.O., 1994), presumably by introductions of infected *O. edulis* from the west coast.

Between 1986 and 1992, massive mortalities of over 90 percent of dredge oysters (*Ostrea chilensis)* were recorded in a wild fishery in Foveaux Strait in New Zealand (Hine, P.M. *et al.*, 2001). The mortalities were found to be associated with a new *Bonamia* species *Bonamia exitosa*. There is some evidence to suggest that *B. exitosa* may also be present in Europe (Abollo, E. *et al.*, 2008). More recently, the importation of European Abalone (*Haliotis tuberculata*) from Ireland to Spain in 2004 resulted in the detection of a new haplosporidian *Haplosporidium montforti* (Azevedo, C. *et al.*, 2006). The affected abalone underwent mortality rates of up to 100% in the six months following deployment in Spain (Balseiro, P. *et al.*, 2006).

1.5.2 Haplosporidians in Western Australia.

Four haplosporidian parasites have been detected in Western Australia.

Bonamiosis in flat oysters (Ostrea angasi).

The aquaculture of flat oysters (*Ostrea angasi*) was attempted in Albany on the South Coast of Western Australia in the 1990s (Anon, 2004). However, the project was abandoned due to mortalities caused by *Bonamia* sp. (Anon, 2004). This incident indicates the potential damage haplosporidian parasites are capable of inflicting to fledgling aquaculture industries.

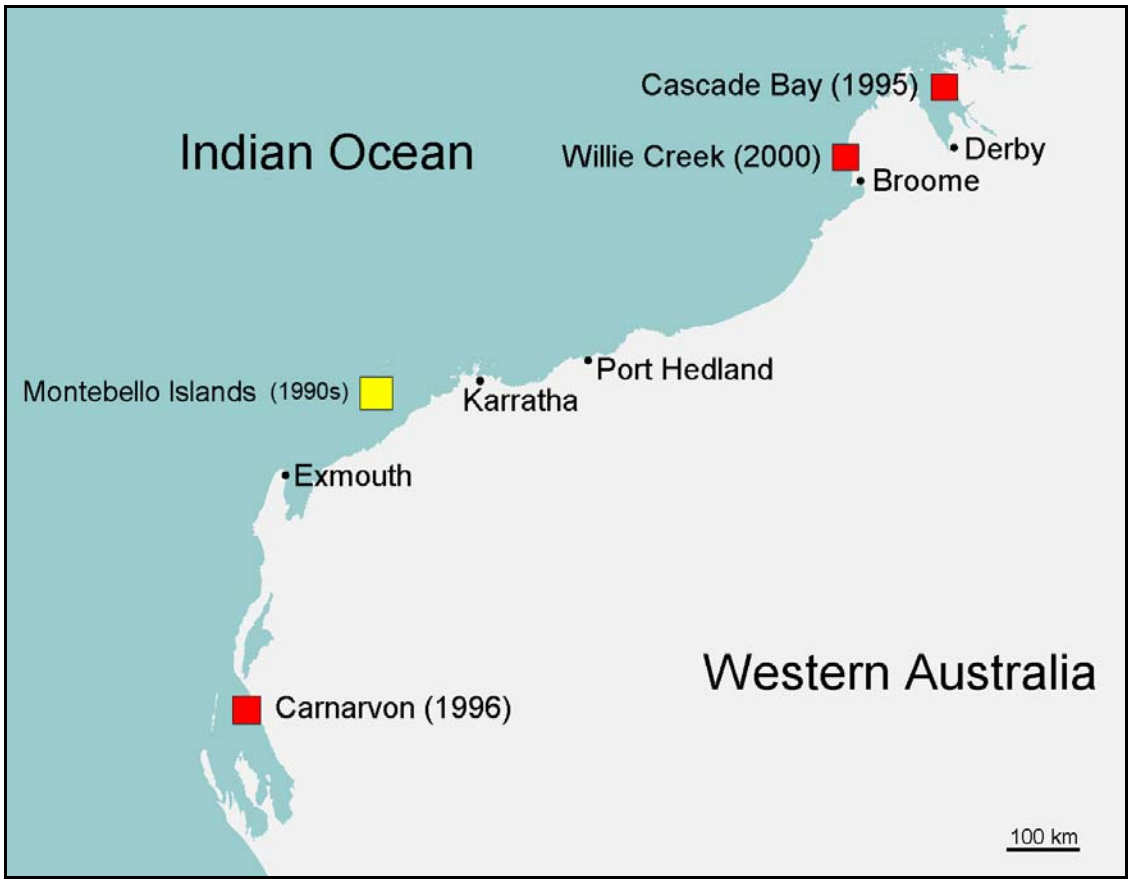


Figure 1.2 Map of north Western Australia indicating the locations of 3 past haplosporidian outbreaks in pearl oysters at Cascade Bay (1995), Carnarvon (1996), Willie Creek (2000) and the location of the haplosporidian outbreak in rock oysters from the Montebello Islands (1990s).

Bonamia roughleyi in Sydney Rock oysters

Bonamia roughleyi is believed to be the aetiological agent of Winter Mortality in Sydney Rock Oysters (*Saccostrea glomerata*). *Bonamia roughleyi* has also been detected in Carnarvon, Western Australia infecting Sydney Rock Oysters. It has not been associated with widespread mortalities perhaps because of warmer water temperatures. The relationship between the Western Australian isolate of *B. roughleyi* and its eastern counterpart is unknown. There have also been sporadic reports of *B. roughleyi* like infections in pearl oysters (Humphrey, J.D., Norton, J.H., Jones, J.B., Barton, M.A., Connell, M.T., Shelley, C.C. and Creeper, J.H., 1998; SCFH, 1993). A single infected pearl oyster was detected in Carnarvon in 1992 (Norris, R., 1996). Although the exact nature of this infection as not been determined.

Haplosporidosis in rock oysters (Saccostrea cucullata).

An undescribed haplosporidian species also occurs in rock oysters (*Saccostrea cucullata*; Hine and Thorne, 2000). This parasite was first recognised after energy companies on the north-west shelf of Western Australia reported high mortalities in oysters in the early 1990s. The companies involved were using rock oysters for pollution monitoring (Anon, 1992; Hine, P.M. and Thorne, T., 2000). The parasite was morphologically described by Hine and Thorne (2002) who tentatively placed the parasite in the genus *Haplosporidium* (Hine, P.M. and Thorne, T., 2002). The identified haplosporidian was associated with extensive mortalities (up to 80%) in the host species and would likely prove an impediment to any attempts at tropical rock oyster aquaculture (Anon, 2004).

Haplosporidosis in pearl oysters (Pinctada maxima).

An undescribed *Haplosporidium* sp. infection of pearl oysters, *Pinctada maxima*, has been detected on at least three occasions (Humphrey, J.D., Norton, J.H., Jones, J.B., Barton, M.A., Connell, M.T., Shelley, C.C. and Creeper, J.H., 1998; Jones, J.B. and Creeper, J.H., 2006). The locations of the infections are shown in Figure 1.2. On each of these occasions, the parasite was detected in pearl oyster spat less than 12 mm in size (Jones, J.B., 2006). While no apparent mortalities were observed, the almost total replacement of the digestive gland epithelium with spores suggests that the infected oysters would not have survived (Figure 1.3; Hine and Thorne 1998). In any case, mortalities directly resulting from the parasite would be difficult to isolate against the relatively high levels of background mortality present in spat. The prevalence of infection determined histologically in the Carnarvon outbreak was 4% (6/150) and in Cascade Bay in King Sound it was 4.7% (7/150). However, when the oysters from the latter outbreak were culled 15 days later the prevalence of infection had increased to 10% (Jones, J.B. and Creeper, J.H., 2006). At the Willie Creek outbreak the prevalence of infection was estimated to be 1.3% (2/150). The organism is considered to represent a serious potential threat to the pearling industry (Humphrey, J.D. and Norton, J.H., 2005).

In addition, haplosporidian-like bodies have also been detected in mature oysters within the Northern Territory and in Western Australia (Humphrey, J.D., Norton, J.H., Jones, J.B., Barton, M.A., Connell, M.T., Shelley, C.C. and Creeper, J.H., 1998). These bodies were derived from oyster populations undergoing mortalities on one occasion from the Northern Territory.

It is certain that many more haplosporidians remain to be discovered, especially as more aquatic species are subject to aquaculture or the environmental stresses associated with economic activity. Because of the long isolation of the Australian continent it is also likely that many of these parasites will prove to be endemic.

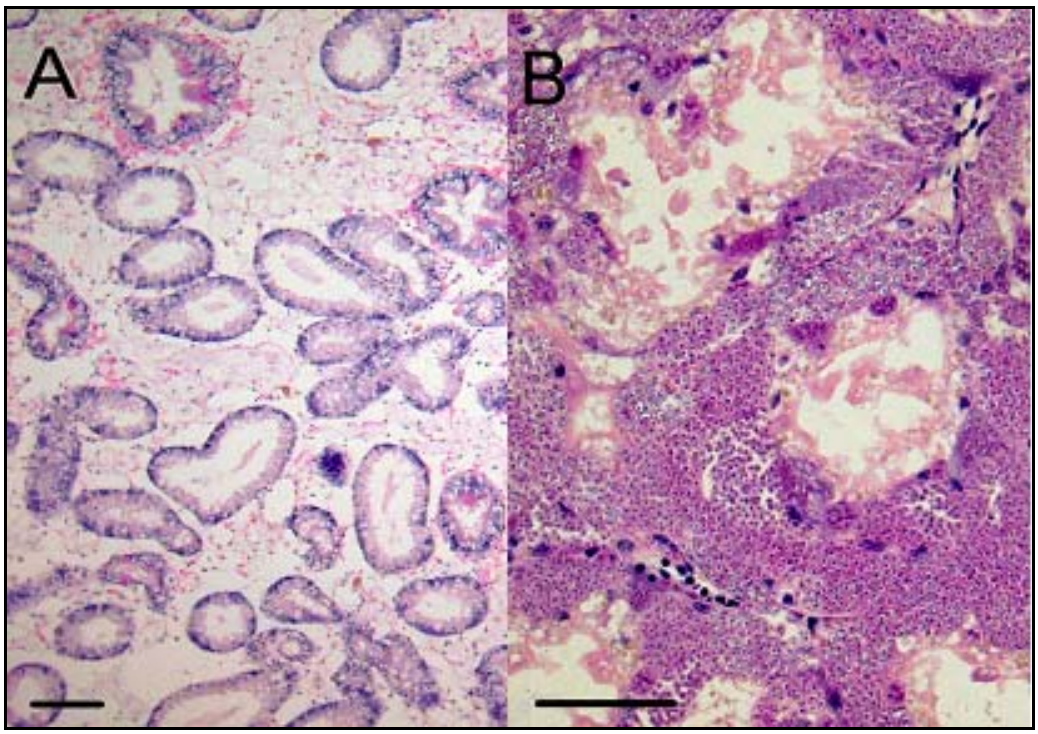


Figure 1.3 Hematoxylin and Eosin (H&E) stained microscope slide of two oyster digestive glands. (A) is an uninfected gland showing intact digestive tubule epithelium. Scale bar represents 150 µm. (B) is the digestive gland of a pearl oyster infected with *Haplosporidium* spp. parasites. Scale bar represents 75 µm. The digestive tubule epithelium has been invaded although it still retains its circular structure.

1.6 The current management of the *Haplosporidium* sp. in Western Australia.

On each of the three occasions where a *Haplosporidium* sp. infection has been detected in pearl oysters the entire batch of oysters was destroyed (Jones, J.B., 2006). This policy has caused a considerable financial loss to the industry. Currently, the policy of the Western Australian Department of Fisheries is to quarantine movement of pearl oysters where an infection has been detected (Jones, J.B., 2006). Since the *Haplosporidium* sp. is only known to affect pearl oyster spat, the current policy would result in substantially less spat being moved to pearling leases for grow out if the parasite became widespread. The consequence of this policy, from an industry perspective, results in fewer oysters being seeded and a smaller harvest of pearls in the following years.

1.7 The requirement for information regarding the infection of *Pinctada maxima* by a *Haplosporidium* sp. and the haplosporidian infecting rock oysters *Saccostrea cucculata*.

There are two major reasons for studying the haplosporidian parasites of northern Western Australia. Firstly, from an academic perspective, information regarding the haplosporidians is important in assessing the taxonomic and phylogenetic status of this group. The haplosporidians constitute a major group of molluscan pathogens which cause a considerable level of mortality to oyster stocks worldwide. Research into any haplosporidian increases the overall understanding of the group as a whole. Secondly, from a practical point of view, information regarding the haplosporidians has important implications for the management of the pearling industry in Australia. This point is particularly relevant for a number of reasons. Information regarding these

parasite species can be used to determine if the haplosporidians are a genuine threat to pearl oyster stocks, and confirm that neither parasite is *H. nelsoni.* This organism is a dangerous pathogen and is notifiable under OIE guidelines. In addition, because of the precautionary approach adopted by authorities, the detection of any *Haplosporidium* results in the quarantine of a considerable number of valuable pearl oysters and, consequently, results in a considerable financial loss to the industry. Assessment of the threat posed to the pearling industry by the parasite will allow a more informed management policy to be developed by the WA Department of Fisheries for dealing with the parasite.

The primary objectives of this study are to describe the haplosporidian parasites of northern Western Australia. Appropriate molecular, pathological and electron microscopy techniques are to be used. The specific aims are to use these methods to:

1) Detect the haplosporidian parasite in rock oysters from north Western Australia by successful amplification of the SSU region of the parasite's rRNA gene and provide evidence of the identity and tissue localization of this parasite by using *in situ* hybridisation (ISH). Any parasite detected can be compared to the parasite described by (Hine, P.M. and Thorne, T., 2002) using the molecular tools developed in this study.
2) Suggest a potential PCR assay for the rock oyster parasite and commence an assessment of its sensitivity and specificity. In addition, two *in-situ* hybridisation assays are also suggested and assessed. Both molecular methods are compared to histology.
3) Determine whether the detected parasite was present in past haplosporidian outbreaks in pearl oysters using the PCR and ISH assays developed in (2) above.

4) Describe the spore ornamentation of the haplosporidian in pearl oysters using both scanning and transmission electron microscopy and compare the results to other haplosporidians and the molecular data obtained.
5) Describe the spore ornamentation of the rock oyster parasite using both transmission and scanning electron microscopy and compare the results to the pearl oyster haplosporidian, other haplosporidians and the molecular data obtained
6) Use the morphological data obtained in (4) to assess the specificity of the molecular tests developed in (3) above.
7) Attempt to infect pearl oysters and rock oysters with the parasite by co-housing infected rock oysters with either pearl oysters or rock oysters with or without potential intermediate hosts and by deploying spat to a site where the haplosporidian parasite had been detected in the past; Cascade Bay. An attempt will also be made to locate the parasite(s) from rock oysters and tropical oysters obtained from the sites where pearl oysters became infected.

Chapter 2 General Site Observations and Sampling Methods.

Fresh samples of haplosporidians were required for the success of this project. However, both haplosporidians had not been detected for five or more years and then only intermittently and often at low levels of prevalence. This meant the sampling methods employed were of vital importance to the project. This chapter describes the sampling methods and sampling locations used in the study to detect haplosporidian parasites at a presumed low level of prevalence.

2.1 Introduction

Potential pathogenic organisms are often present at low levels of prevalence (Hine, P.M. and Thorne, T., 2000) or they are cryptic and therefore not easily diagnosed using histopathology. Molecular diagnostic assays are sensitive and offer an alternative method for the detection of cryptic parasites since they can target the DNA of a pathogen regardless of the life history stage present. However, too often samples are routinely fixed solely in formalin without regard for confirmation diagnostics or future research. Formalin fixation allows the samples to be processed for histological examination but results in the cross-linking of histones and fragmentation of sample DNA making amplification of sequences larger than 300 bp and subsequent molecular analysis difficult (Paabo, S., 1990) especially where the parasite is undescribed. For molecular assays such as PCR to be used in aquaculture, frozen or ethanol stored samples need to be taken.

Strict controls are often imposed on aquaculture industries to limit translocation of parasites that may represent an economic threat to industry. One such measure includes a requirement for formalin fixed samples to be obtained for histological examination prior to movement of a cultured species between management zones. One way to increase the research and diagnostic capabilities of management authorities would be to request replicate ethanol stored or frozen samples to be obtained at the same time and from the same individuals that are sampled through formalin fixation. The replicate samples can be held by the aquaculture enterprise. If the stock appears healthy after a histological examination then the replicate samples can be disposed of. This way a first class sampling regime can be obtained for

minimal cost that incorporates molecular capabilities. This has a number of advantages.

Firstly, replicate samples allow confirmation of a diagnosis made by histology where appropriate molecular diagnostic tests already exist. A diagnosis based on histopathology can be confirmed through PCR. This capability is important were a pathogen is difficult to definitively diagnose based on parasite species morphology criteria alone. If molecular diagnostic assays such as PCR are to be used in aquaculture then both formalin fixed and ethanol stored or frozen samples are required since molecular assays such as PCR require visual confirmation (Burreson, E.M., 2000; Kleeman, S.N. and Adlard, R.D., 2000). Diagnostic methods such as histology and *in-situ* hybridisation that allow visual confirmation require formalin fixation. Replicate sampling will allow confirmation of the diagnosis down to the infected individuals.

Secondly, replicate sampling allows considerably more research to be targeted towards any emerging parasite. Molecular techniques allow the sequencing of an appropriate gene region of a parasite species detected in histology and therefore allow phylogenetic analyses and the development of appropriate diagnostic assays. This is an important feature in the marine environment where many potentially serious pathogens remain to be discovered or are present at a low level of prevalence (Hine, P.M. and Thorne, T., 2000; Jones, J.B. and Creeper, J.H., 2006). This is especially the case in Western Australia where the age and isolation of the Australian continent would suggest many of these pathogens would be unique.

Thirdly, replicate sampling allows comparison of the sensitivity and specificity between diagnostic techniques such as a molecular assay compared to histology and therefore allows some preliminary assessment of its reliability. A number of samples of the pathogen over the full length of its range are required for the validation of molecular assays. The routine collection of ethanol stored or frozen samples (along with replicate formalin fixed samples) would facilitate this process especially where the parasite exists at low prevalence and is therefore difficult to sample independently.

Finally, but perhaps most importantly, replicate sampling reduces the time taken for the development and validation of a molecular assay, since research can be well advanced for the reasons mentioned above. Also, histologically positive animals can be used to develop appropriate molecular assays rather than relying on a random assortment of animals from the affected batch that may or may not have the infection depending on the prevalence of the pathogen. This point is especially relevant to pathogens that may be present at very low prevalence. The long lag times required to develop and validate a molecular assay, combined with the potentially low levels of sensitivity of histology against some types of cryptic pathogens (such as viral agents) may mean an emerging pathogen can become widespread before detection.

Industry often finds it difficult to tolerate blanket bans on the movement of a cultured species while appropriate molecular assays are developed. However, once such a parasite becomes established then it is almost impossible to eradicate from the aquaculture setting. The keys to prevent the spread of an emerging pathogen are rapid diagnosis and the prevention of transport of the affected stock. The use of replicate samples can aid the detection of emerging pathogens, speed research into

their significance and aid in the development of appropriate molecular diagnostic assays for their future detection. Molecular assays can play a much more significant role in the control of aquatic diseases if they are developed before a pathogen becomes established in industry.

The pearl oyster *Haplosporidium* sp. has only been detected three times in the past ten years. The prevalence of *Haplosporidium* sp. in rock oysters is difficult to accurately determine. Multiple attempts to obtain the rock oyster *Haplosporidium* sp. following the development of a specific probe for *H. nelsoni* were unsuccessful (Hine, P.M. and Thorne, T., 2002). Only formalin fixed paraffin embedded samples were available for both parasite species. Consequently, the sampling strategy employed was of vital importance to obtaining new *Haplosporidium* samples suitable for molecular analysis and the overall success of the project.

2.2 Materials and Methods.

2.2.1 Sampling regime and details of collection sites for the pearl oyster Haplosporidium sp.

Formalin fixed paraffin embedded tissue from pearl oyster spat infected with *Haplosporidium* sp. were obtained from the Western Australian Department of Fisheries. These samples included all three of the pearl oyster *Haplosporidium* sp. infections that have been detected (Figure 1.1).

Table 2.1 Date, Location and number of pearl oysters collected and used in this study.

Pearl oyster:	Location	Latitude	Longitude	Date	Method	n Formalin fixed	n Ethanol stored
	Cascade bay	-16.59' S	123.54' E	14/3/06	Spat deployed on longline	171	152
				30/5/06	"	120	120*
				22/9/06	"	124	124*
				15/4/07	"	122	122*
	Willie creek	-17.42' S	122.10' E	8/05/2006	Sample wild oysters	20	20*

* indicates replicate samples from the same individual oysters as fixed in formalin.

In addition to the samples mentioned above, an attempt to procure fresh *Haplosporidium* sp. from pearl oysters was made by sampling the sites that had previously produced an infection (Figure 1.1 Table 2.1). This strategy would not only provide fresh material for molecular assays (if successful) but would also help ascertain whether the parasite is still present at these locations. This information would allow some assessment of whether the parasite's occurrence in pearl oysters was a rare event at each of the sites or whether this parasite is persistent in the environment but remains localised due to the actions of the pearling companies and

the WA Department of Fisheries. However, the occurrence of the *Haplosporidium* sp. is a sensitive issue for lease holders and this fact limited the number of fresh pearl oysters that could be acquired. Pearl oyster samples were not obtained from the Carnarvon site due to the reluctance of the lease holder and the cost of deploying spat to the site.

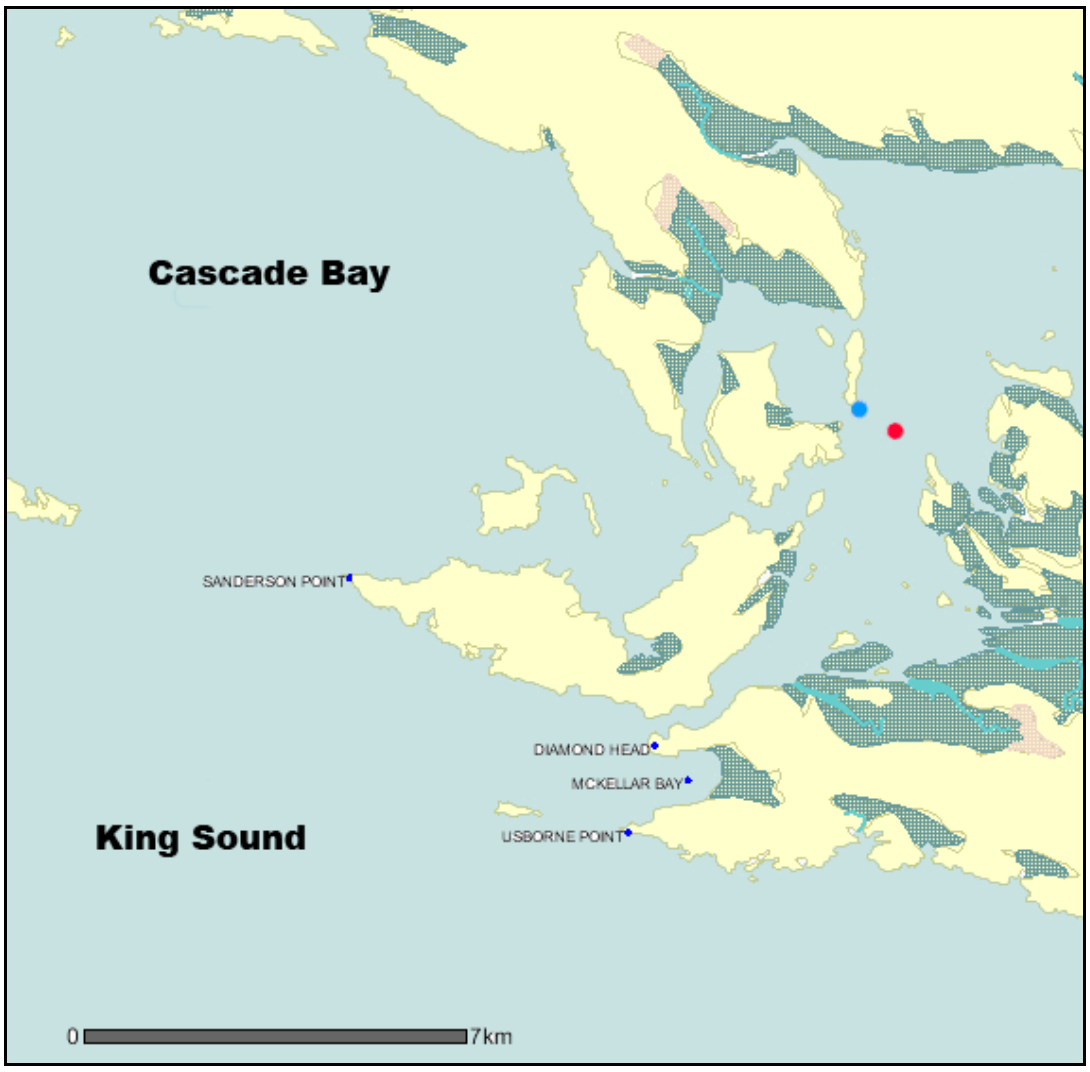


Figure 2.1 Map of Cascade bay indicating the position of the deployed pearl oyster spat (●) and the site where the Cascade bay rock oyster sample was obtained (●). The green areas indicate the intertidal zones. Map outline was obtained from WALIS (2006).

2.2.1.1 Pearl oyster sampling at Cascade Bay

Cascade Bay, in King Sound, was subject to a *Haplosporidium* sp. infection in 1995. This site was sampled by deploying approximately 6000 pearl oyster spat in February 2006 with the assistance of Blue Seas Pty Ltd (Figure 2.1). The spat were obtained from the Broome pearl oyster hatchery and were approximately 5 mm in size. As a whole, the area consists predominately of mangroves and rocky outcrops. The site is within 200 m of where the original infection occurred (Brown, W., 2006). The deployment site is within a narrowing of the bay (Figure 2.1) and would therefore be subjected to a high volume of water flow. The co-ordinates of the deployment site was latitude of -16.59' S with a longitude of -123.54' E (Table 2.1). The exact date and number of samples obtained for each sampling trip is indicated in Table 2.1.

The pearl oyster spat deployed at Cascade bay were suspended in the water from a longline in plastic panels at an approximate depth of two meters in a similar manner to the oysters that were infected in 1995 (Figure 2.1). The depth of the site was 12 m with a substrate consisting of mud.

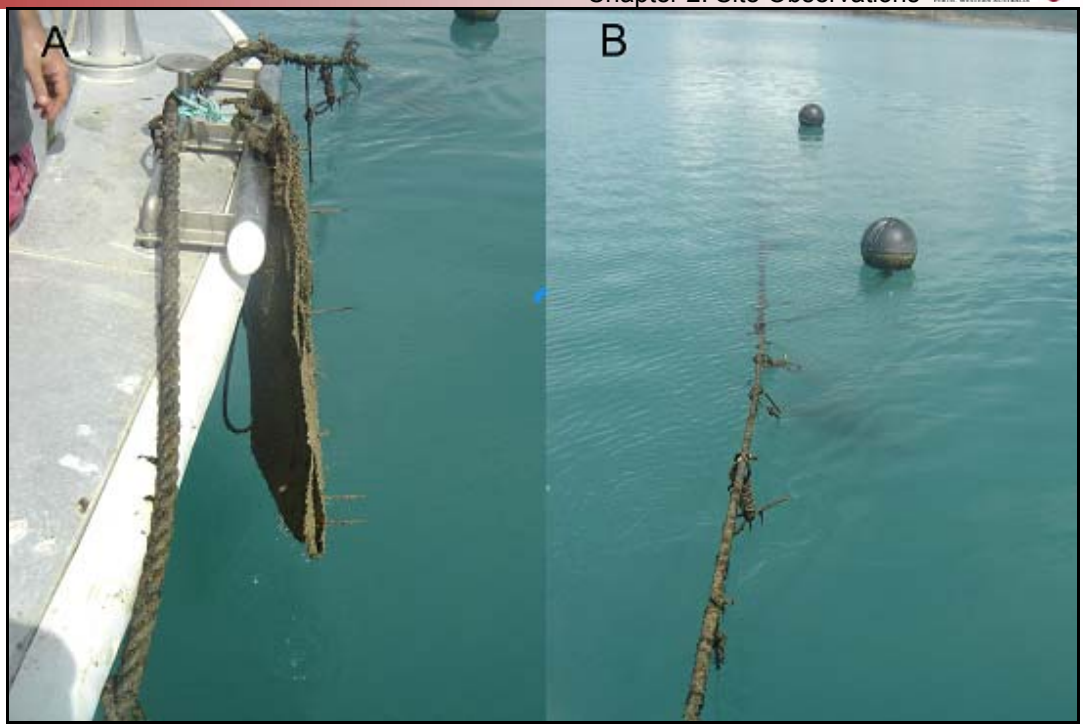


Figure 2.2 The deployment of pearl oyster spat on a longline in Cascade Bay, King Sound. Picture A shows the panel that the pearl oyster spat were deployed in. Picture B shows the longline the panels of spat were suspended from.

2.2.1.2 Pearl oyster sampling at Willie Creek.

Sampling also occurred at the Willie Creek lease site in May 2006. The lease runs half a mile either side of 17° 42'.12 S 122° 10' 33 E on the northern edge and 17° 43'.21 S 122° 10'.35 E on the southern side (Figure 2.3). This site was subject to an infection in February 2000. Pearl oyster spat could not be deployed to the site due to the reluctance of the current lease holder. Consequently, the site was dived for pearl oysters in May 2006 with the assistance of Kailis Pearls Pty Ltd and Paspaley Pearls Pty Ltd.

The lease area consists of an area of garden bottom with exposed rocks and coral while the remainder is covered in a silty mud with numerous bivalve species.

Figure 2.3 Map indicating sampling sites for the Willie Creek rock oyster (●) and pearl oyster samples (●). Source of map outline was WALIS (2006).

2.2.2 Sampling regime and details of collection sites for rock oyster Haplosporidium sp.

The rare occurrence of the *Haplosporidium* sp. in pearl oysters is suggestive of a parasite that may be naturally occurring in an alternative host. One possible candidate was the *Haplosporidium* sp. occurring in rock oysters since it had already been reported twice previously in this species (Anon, 1992; Hine, P.M. and Thorne, T., 2002). In order to assess this possibility, samples of rock oysters were obtained from the intertidal zones of four locations (Bluebell Island, West bay, Chartreude bay and Crocus Island) at the Montebello Islands in July 2005 (Figure 2.4). Samples of rock oysters were also obtained from each of the positive pearl oyster sites (Cascade bay, Willie creek, and Carnarvon; Figure 1.1; Table 2.1). Archived histologically negative samples of rock oysters from Carnarvon and tropical oysters (*Saccostrea echinata*) from Koolan Island were obtained from the archives of the Western Australian Department of Fisheries.

Table 2.2 Location and number of rock oysters and tropical oysters collected and used for this study

	Location	Latitude	Longitude	Date of collection	Method	n
Rock Oyster:	Montebello Islands	-20°.4' S	115°.53' E	July 2005	Removed from intertidal rocks	291
	Cascade bay	-16°.59' S	123°.54' E	March 2006	Removed from intertidal rocks	72
	Quondong point near Willie creek	-17°.66' S	122°.18' E	May 2006	Removed from intertidal rocks	65
	Carnarvon	-24°.53' S	113°. 40' E	1995	Archived samples	36
Tropical Oyster	Koolan Island	-16° 60' S	123° 45' E	1994	Archived samples	27

Formalin fixed paraffin embedded tissues from positive *Haplosporidium* sp. rock oyster infections were also obtained from the Western Australian Department of Fisheries. This infection was described by Hine and Thorne (2002).

2.2.2.1 Rock oyster sampling at the Montebello Islands

The Montebello Islands were selected since at least two infections of rock oyster *Haplosporidium* sp. had been detected there (Anon, 1992; Hine, P.M. and Thorne, T., 2002). Four sites were chosen for sampling since the oysters present at each appeared to be undergoing a higher than usual mortality relative to the surrounding areas (Personal observations; Figure 2.4; Figure 2.6).

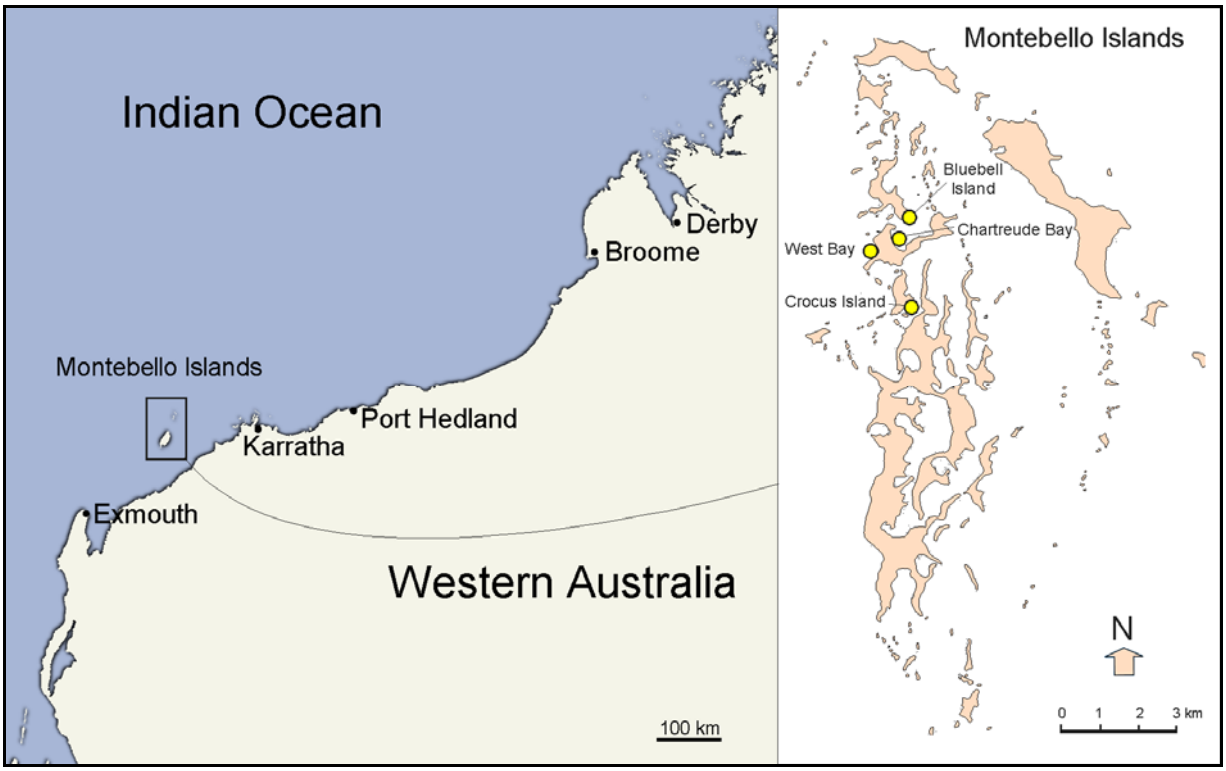


Figure 2.4 Map of Western Australia with the Montebello Islands shown in the insert (WALIS, 2006). The four sampling locations are indicated as a yellow dot.

Two hundred and ninety one rock oysters were removed from the intertidal zones at each site using a hammer and chisel (Table 2.3). The numbers from each of the four sites are indicated within Table 2.3.

Figure 2.5 shows the West bay sample site (Figure 2.5). The Montebello Islands are characterised by low lying intertidal rocks interspaced by beaches and mangroves. This site was typical of all four sample sites.

Figure 2.5 Picture of West bay rock oyster sample site at the Montebello Islands. This site was typical of the four sites from which rock oysters were obtained.

Table 2.3 The total number of rock oysters collected from each of the four Montebello Island sample sites.

Location:	**n**
Bluebell Island	92
West bay	97
Chartreude bay	58
Crocus Island	44
Total	**291**

The four sample sites were chosen because the oysters at these sites appeared to be suffering from a higher level of mortality than the surrounding areas. Figure 2.6 shows a number of recently deceased oysters although the cause is not certain.

Figure 2.6 Picture of rock oysters at the West bay sample site in the Montebello Islands. The site appeared to have higher levels of oyster mortality present than the surrounding areas although the cause is not certain.

Sampling at the Montebello sites was carried out with the assistance of Morgan Pty Ltd.

2.2.2.2 Rock oyster sampling at Cascade bay, Willie creek and Carnarvon sites

Rock oysters were sampled from intertidal areas as close as possible to the sites where the pearl oyster *Haplosporidium* sp. had been previously detected. These samples were obtained so that the presence of the parasite in rock oysters at these sites could be confirmed. In accordance with these aims, seventy two individuals were collected from the Cascade bay site (Table 2.4) approximately 400 m from the pearl oyster spat deployment site (Figure 2.1). Sixty five rock oysters were sampled from the Quondong point site approximately two kilometres from the Willie creek lease site and thirty six rock oysters were obtained from Oyster creek. Oyster creek is

approximately 2 km from the site of the Carnarvon outbreak (Table 2.4). The Carnarvon rock oysters and Koolan Island tropical oysters were obtained as archived samples from the Western Australian Department of Fisheries. These samples were formalin fixed and paraffin embedded and were collected in 1994 (Koolan Island sample) and 1995 (Carnarvon sample).

Table 2.4 Number of rock oysters taken from Cascade Bay, Willie Creek, Carnarvon and Koolan Island.

Location	Oysters obtained
Cascade bay	72
Quondong point (near Willie Creek)	65
Carnarvon#	36
Koolan Island*	27
Total	200

* Koolan island sample consisted of archived tropical oysters (*Saccostrea echinata*). # Carnarvon samples consisted of archived rock oyster samples.

2.3 Processing of pearl oyster and rock oyster samples.

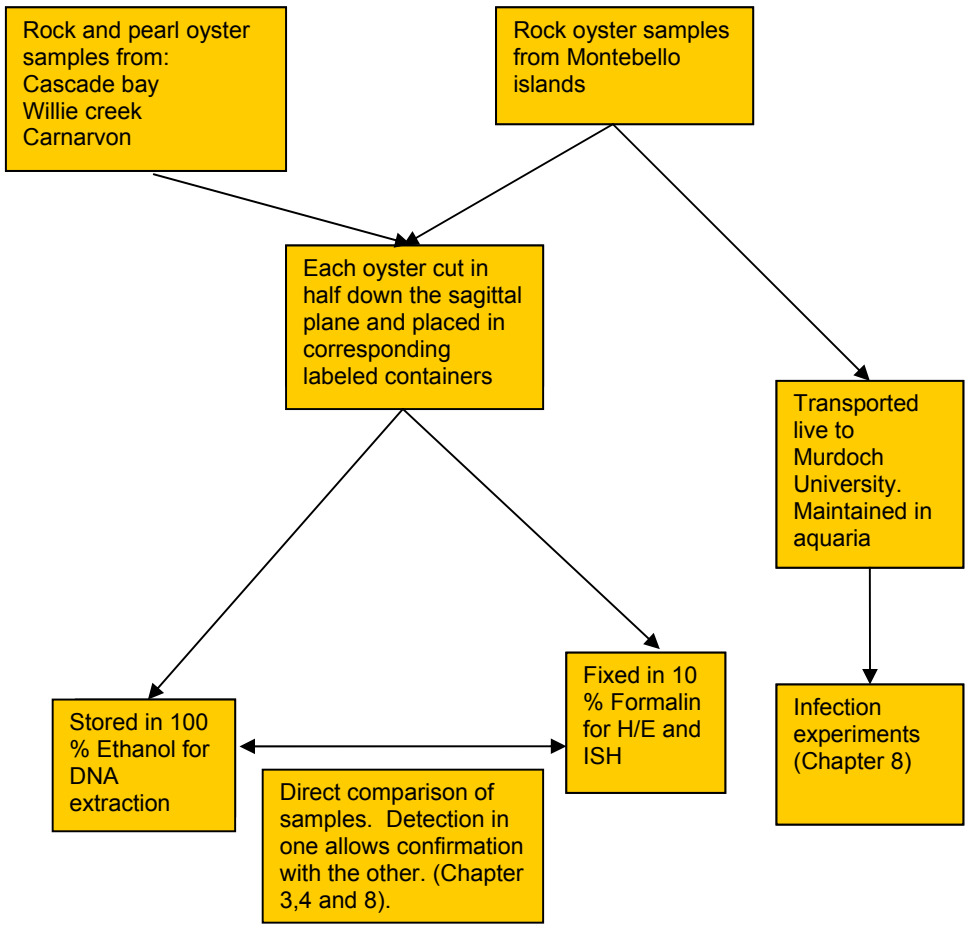


Figure 2.7 Flow diagram indicating the treatment of samples obtained from the Cascade bay, Willie creek Carnarvon and Montebello Island sample sites.

An outline of the treatment of samples from each of the sites is indicated in Figure 2.7

2.3.1 Processing of pearl oyster samples.

2.3.1.1 Cascade bay pearl oyster spat.

Since approximately 6000 pearl oyster spat had been deployed to Cascade bay, a number of sampling trips occurred. On each of these occasions approximately one

hundred and twenty spat were removed from the longline and placed in 10% sea water buffered formalin for 24 hours. Replicate sampling was performed for all sampling trips except the first so samples of the same individual oysters were also preserved in ethanol (Table 2.2). The number of samples was viewed as adequate since the OIE list this volume to be capable of detecting a 2% infection with 95% confidence (OIE, 2006). A similar number is required for health certification by the Western Australian Department of Fisheries. The deployment size also allows for a sizeable natural mortality which is expected when pearl oyster spat are transferred from the sheltered conditions of the hatchery to the ocean.

2.3.1.2 Willie Creek and Carnarvon pearl oyster samples.

Sampling at the Willie Creek site resulted in the capture of 20 adult pearl oysters. The pearl oysters were removed from their shells and cut down the sagittal plane so that a piece of gill and digestive gland were present in each oyster section. The first half from each oyster was fixed in 10% sea water buffered formalin for 24 h while the other half was stored in 100% ethanol.

2.3.2 Processing of rock oyster samples.

2.3.2.1 Rock oyster samples from the Montebello islands.

A total of 291 rock oysters were obtained from the Montebello Islands. Of these 198 were transported alive to Murdoch University for cross infection trials (Table 2.5). The numbers of oysters transported alive from each of the sample sites are indicated in Table 2.5.

Table 2.5 The number of rock oysters obtained from each of the different sampling sites in the Montebello islands and their treatment.

Location:	Number with tissue fixed in 10% Formalin and 100% Ethanol	Number transported to Murdoch University alive.	Total
Bluebell Island	24	68	92
West bay	29	68	97
Chartreude bay	23	35	58
Crocus Island	17	27	44
Total	93	198	291

Foam boxes were used to transport the live rock oysters. Holes were cut in the lid and damp newspaper was wrapped around freezer blocks. These were then placed in the bottom of the box. Rock oysters were placed on top of the freezer block with the cup side down. The total time the oysters were out of the water to the aquaria was approximately 14 h. The method used to maintain the rock oysters is described in Chapter 5.

The remaining oysters (93) were processed by removing the soft tissue from each oyster. This was then cut into two pieces along the sagittal plane. A sagittal section was used so that a piece of gill tissue and digestive organ was present in each oyster section. One piece was placed into a labeled container and fixed in 10% sea water buffered formalin for 24 h. This tissue was embedded in paraffin on return to Murdoch University and examined using conventional histological techniques or *in situ* hybridization (Chapter 3). The other piece was placed into another corresponding labeled container and fixed in ethanol for genomic DNA extraction and subsequent PCR analysis (Chapter 3). By using this method it was possible to cross reference oyster samples so that a positive result by traditional histology or by molecular tests

could be used to optimise the alternative method. The numbers from each of the four sites are indicated within the table 2.5.

2.3.2.2 Rock oyster samples from Cascade bay, Willie Creek Carnarvon and Koolan Island.

For the remaining sites, rock oysters were removed from intertidal locations as close as possible to the sites of previous pearl oyster *Haplosporidium* sp. infections using a hammer and chisel. One half of each oyster was stored in ethanol for DNA extraction while the other was fixed in 10% sea water buffered formalin. The numbers of oysters fixed by each method from each of the sites are given within Table 2.6. The Carnarvon and Koolan Island samples were obtained as archived formalin fixed paraffin embedded samples from the Western Australian Department of Fisheries.

Table 2.6 Numbers of rock oyster samples taken from Cascade bay, Willie creek and Carnarvon and the method used to store them.

Location	100% Ethanol	10% Formalin	Total
Cascade bay	72	72	72
Quondong Point near Willie Creek	62	62	62
Carnarvon	-	36	36
Koolan Island*	-	27	27

* Koolan Island samples consisted of tropical oysters.

Chapter 3 : Molecular characterisation of a haplosporidian parasite infecting rock oysters *Saccostrea cucullata* in north Western Australia.

This chapter describes the detection and molecular characterisation of a haplosporidian parasite infecting rock oysters (*Saccostrea cucullata*) from the Montebello Islands in north Western Australia.

3.1 Introduction

Many haplosporidians are cryptic and are likely to be missed in histology sections. These parasites are also often present at a very low prevalence and there are a variety of morphologically similar species that can be difficult to differentiate during cytological or histological diagnosis. Attempts by others to diagnose the haplosporidian parasite at the Montebello Islands following the development of a specific probe for *Haplosporidium nelsoni* were unsuccessful despite several attempts (Hine, P.M. and Thorne, T., 2002).

Detection of the parasite and sequencing of its rRNA gene would allow speciation of the parasite and confirm that it is not a described species. Sequencing of the parasite's small ribosomal subunit (SSU) rRNA gene would also allow the development of molecular assays for the parasite and thus gain some understanding of the parasite's biology and why it has been so difficult to detect in the past despite causing extensive mortalities in the host species (up to 80%; Hine *et al*.,2002). The development of sensitive and specific molecular assays, utilizing polymerase chain reaction and *in situ* hybridization, provides a means to detect the cryptic life stages of haplosporidian parasites and to identify them. The SSU region of the parasite's rRNA gene can be used for this purpose since it has both conserved and variable regions.

This chapter describes the detection of a haplosporidian parasite in rock oysters from north Western Australia by successful amplification of the SSU region of the parasite's rRNA gene and provides evidence of the identity of this parasite by using *in situ* hybridisation (ISH). The parasite detected is compared to the parasite described by Hine and Thorne (2002) using the molecular tools developed in this study.

3.2 Materials and methods

The outline of the strategy adopted to target the haplosporidian parasite parasitizing rock oysters is indicated in Figure 3.1.

3.2.1 Oyster sampling

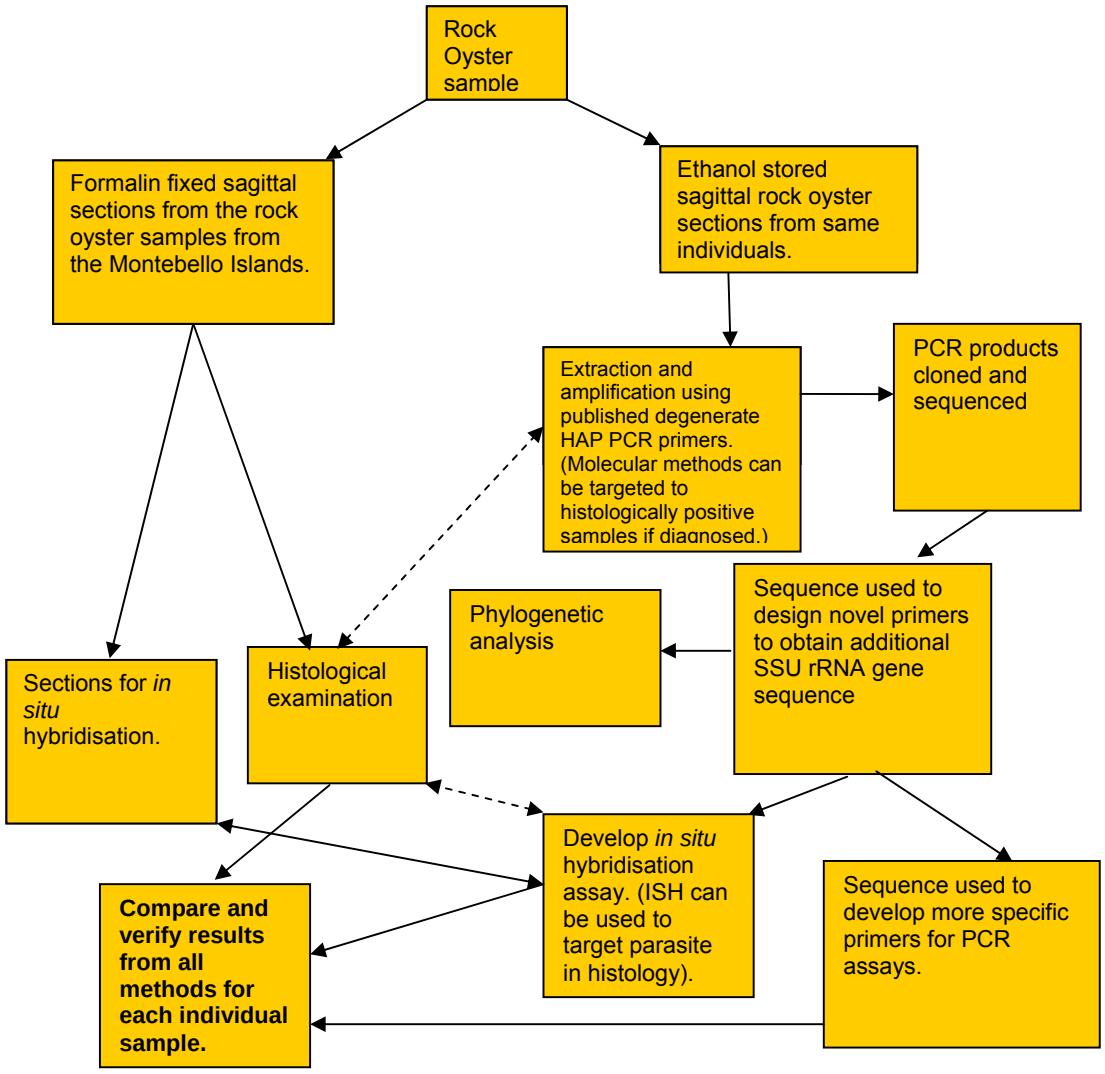


Figure 3.1 A flow diagram indicating the strategy adopted to target the haplosporidian parasite in the rock oyster samples from the Montebello Islands. This integrated approach can only be employed when using a replicate sampling regime as described in Chapter 2. Dotted line indicates the results of one diagnostic test can be compared to the other for each individual oyster.

Adult rock oysters were obtained from the Montebello Islands (latitude -20.4' S longitude 115.53' E) on the north-west coast of Western Australia in July 2005 as described in Chapter 2. In order to assess the specificity of the ISH assay unstained *Haplosporidium costale* and *Minchinia teredinis* sections were obtained from the Virginia Institute of Marine Science (VIMS). Representative histologically positive formalin fixed paraffin embedded rock oysters infected with the parasite described by Hine and Thorne (2002) were obtained from the Western Australian Department of Fisheries for DNA extraction and ISH.

3.2.2 DNA extraction

Two different methods of DNA extraction were used. Genomic DNA from 26 ethanol stored rock oysters was extracted from approximately 5 mg of gill tissue using a Masterpure™ DNA purification kit (Epicentre Technologies, Sydney) and was performed according to the manufacturer's protocol. Briefly, approximately 5 mg of tissue from each individual was homogenized in a labelled 1.5 ml eppendorf tube containing 300 µL of Tissue and Cell Lysis Solution (Epicentre Technologies, Sydney). A 1 µL aliquot of Proteinase K (50 µg/µL; 50 mM Tris – HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 10 mM $CaCl_2$, 0.1% Triton X-100, 1 mM dithiothreitol, 50% glycerol) was added to each tube followed by a brief vortex. Individual samples were subsequently incubated in a 65 °C water bath for 15 minutes, and each tube was vortexed at five minute intervals. This incubation period allowed the Proteinase K to fully digest the tissue. The water bath containing the tubes was then cooled to 37 °C, with a 1 µL aliquot of RNase A (5 µg/µL; 25 mM NH_4OAc (pH 4.5), 50% glycerol)

being added to each tube, followed by vigorous mixing and incubated at 37 °C for 30 minutes. The tubes were then placed on ice for 3 to 5 minutes.

A 150 µL aliquot of MPC Protein Precipitation Reagent (Cat N^{o} MCD 85201, Epicentre Technologies, Sydney) was added to each sample followed by vigorous mixing for 10 seconds. Tubes were centrifuged at 10000 rpm for 10 minutes. The supernatant from each sample was then transferred to a clean labelled 1.5 mL tube and the pellet discarded. To precipitate the DNA, 100% isopropanol (500 µL) was added to each and the tubes inverted 40 times.

The DNA was pelleted by centrifuging the samples at 15 000 rpm for 10 minutes. The supernatant was removed and the resulting pellet washed twice with 500 µL of 75$^{\%}$ ethanol. The ethanol was removed by gently pipetting or pouring off. The tubes were then left to dry overnight. 35 µL of TE buffer (10 nM Tris – HCl (pH 8.0), 1 nM EDTA) was used to resuspend the DNA pellet, which was then stored in a – 20°C freezer.

3.2.3 DNA extraction: archived samples

Genomic DNA was extracted from the formalin fixed paraffin embedded rock oysters by following a freeze thaw extraction method (Anon, 1998). The procedure was performed as follows. For each sample three 12 µm sections were cut from the block and placed in a sterile 1.5ml centrifuge tube. Considerable care was taken to ensure that no DNA could be transferred between samples with each block being processed on separate occasions and new sterile blades being used. The tube was centrifuged at 16100 *g* for 1min in order to pellet the tissue. Two hundred microliters of sterile water with 0.5% v/v Tween 20 was added followed by 10 min boiling. The sample was

then frozen in liquid nitrogen for 10 min. The freeze thaw process was repeated another two times and followed by a 20 min centrifuge at 4500 rpm. The supernatant was removed to a fresh 1.5 ml eppendorf. Another eppendorf containing a 1:5 dilution of the extract was also produced. The quality of the genomic DNA and absence of inhibitory factors from the extractions was verified with bivalve primers 16 R3 5'-GCT GTT ATC CCT RNR GTA-3' and Proto 16_F 5'-AWK WGA CRA GAA GAC-3' (Chase et al., 1998) that target a region of the 18 s rRNA gene.

3.2.4 Amplification by Polymerase Chain Reaction

Originally, degenerate haplosporidian primers (Hap F1 and Hap R3; Table 3.1) were used in the polymerase chain reaction (PCR) to detect a haplosporidian species in ethanol preserved rock oyster tissue. These primers amplify a 344 bp section of the parasite's small subunit (SSU) region of the rRNA gene and were chosen as previous studies, such as those of Renault et al. (2000), have used this primer set to amplify DNA from undescribed haplosporidians. *Haplosporidium nelsoni* DNA was used as a positive control in the initial PCRs. All amplifications were performed on an Eppendorf Mastercycler Gradient Thermocycler.

Each PCR was performed using reaction mixtures that had a total volume of 50 µL and contained reaction buffer (67 mM Tris–HCl, 16.6 mM $[NH4]_2SO_4$, 0.45% Triton X-100, 0.2 mg/ml gelatin, and 0.2 mM dNTP's), 2mM of $MgCl_2$, 40 pmol of each primer, and 0.55 U of *Taq* polymerase and template DNA. Each of the reaction mixtures was subjected to: (i) an initial denaturation phase of 5 min at 94°C, (ii) 35 amplification cycles, each cycle consisting of 30 s of denaturation at 94°C, 30 s of annealing at 48°C, 4 min of extension at 68°C and (iii) a final 7min extension at 68°C. The success

of each PCR was determined by loading a 10 µL aliquot of the PCR products on a 2% agarose gel where it underwent electrophoresis it for 20 min at 46 mA. Detection of the PCR products was performed using ethidium bromide staining.

Table 3.1 Polymerase Chain Reaction primer sequences employed in the study**.**

Primer	Sequence (5' -3')	Position	Reference
Hap F1	gttctttcwtgattctatgma	1144	(Renault, T. *et al.*, 2000)
Hap R3	akrhrttcctwgttcaagayga	1467	(Renault, T. *et al.*, 2000)
Minch F1B	ctcgcgggctcagctt	1295	Present study
Minch R2B	ggcgctttgcagattcccca	1439	Present study
FSSUF	ctcaaagattaagccatgcatgtccaagtata	*	Present study
16 s b	gatcccttccgcaggttcacctac	1682	(Medlin, L. *et al.*, 1988)
In situ probe: SSR 69	agcccaaaaccaacaaaacgtccacatgcg	754	Present study

*FSSUF primer starts at base 15 of most haplosporidian sequences in Genbank.

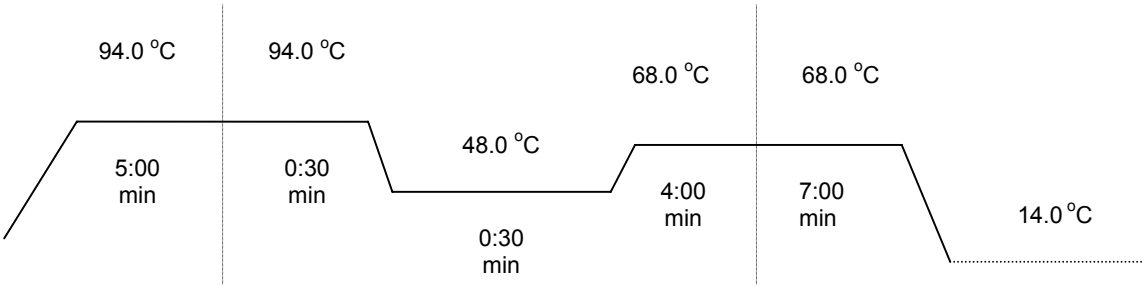


Figure 3.2 Polymerase Chain Reaction (PCR) conditions used for DNA amplification of the SSU rRNA gene utilised in the study. Each of the PCR reactions was subjected to 25 cycles.

The success of each PCR reaction was determined by loading a 10 µL aliquot of the PCR products on a 2% agarose gel which was electrophoresed for 20 min at 46 Amps. Electrophoresed gels were then subjected to approximately 25 min ethidium bromide staining. A P*hi* / *Hae*III Marker (Promega, Sydney) was used as a DNA

standard and viewed using UV light supplied by a Gel Doc ™ (Bio Rad, Sydney). Digital images were viewed and saved by using the Quantity One ™ computer program (Appendix 2; Bio Rad, Sydney). QIAQUICK spin-columns were used to purify the successful PCR amplifications following the manufacturer's protocol (Qiagen, 2000).

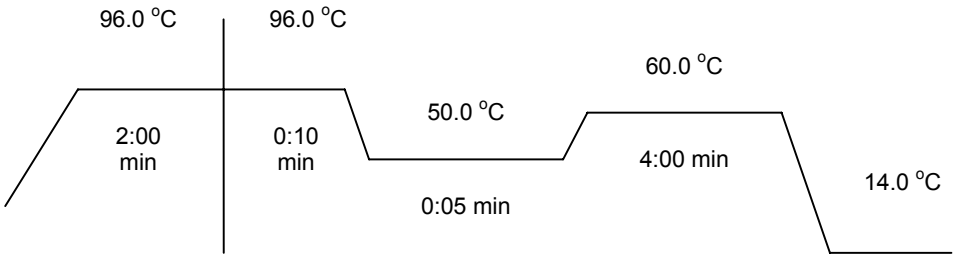


Figure 3.3 Conditions for the cycle sequencing of the cloned PCR products. The PCR reactions were subjected to 25 cycles.

3.2.5 DNA sequencing

To enable sequencing, the PCR products were cloned into a plasmid vector PCR2.1 using the TA cloning kit (Invitrogen Life Technologies) following the manufacturer's protocol. The cycle sequencing of the purified PCR products was completed using Big Dye 3.1 sequencing chemistry. The PCR products were run on polyacrylamide gels in an ABI 377 XL Automated Sequencer (Applied Biosystems, 2001), with reaction mixtures as follows (per tube); *ca* 350 ng template (*ca* 4 µL PCR product), 4 µL dye terminator mix, 1.0 µL ddH_20, 1.6 picomoles (1.6 µL at working solution) of either forward or reverse primer. Each cycle sequencing reaction was performed at half the volume of the manufacturers' recommendations (Applied Biosystems, 2001), so that a significant cost saving could be made. Each reaction was denatured at 96°C for 2 min (Figure 3.3). Each reaction was subjected to 25 reaction cycles, with each cycle

consisting of 10 seconds denaturation at 96°C, five seconds of annealing at 50°C, four minutes of extension at 60°C and finally cooling to 14°C (Figure 3.3).

The sequencing products were cleaned using an ethanol precipitation procedure to exclude unincorporated dye terminators. This process consisted of adding 1 µL of 3M sodium acetate (pH 5.2), 1 µL of 125 mM EDTA and 25 µL of 100% ethanol to each product, followed by a brief vortex and storing on ice for 20 min. The mixtures were then centrifuged at 13 000 rpm for 30 min. The supernatant was discarded using a pipette and the remaining pellet washed with 125 µL of 80% ethanol and then centrifuged at 13 000 rpm for a further five minutes. The supernatant was again discarded, with the final pellet dried in a vacuum manifold for 15 min.

Before automated sequencing, the PCR products were mixed with a loading buffer consisting of 5:1 deionised formamide, 25 mM EDTA (pH 8.0) and 50 mg/mL blue dextran (Applied Biosystems, 2001). The mixtures were then denatured at 95 °C for five minutes and stored on ice until loading. Approximately 0.8 µL of each sample was loaded into wells on a 48 cm well-to-read LongRanger™ polyacrylamide gel and run for about 11 h according to the manufacturer's protocol (Applied Biosystems, 2001).

Three cloned DNA inserts were each sequenced and forward and reverse sequences were aligned and any alignment errors or base misreads were corrected by comparing to the alternative sequence. The primer sequences were removed from each end of the resulting consensus sequence. The position and direction of the rRNA PCR products were verified by BLAST searches of the GenBank database: (http://www.ncbi.nlm.nih.gov/blast/).

Sequences were aligned for further primer development to host 18 s and other haplosporidian SSU sequences using the CLUSTALW algorithm (Thompson, J.D. *et al.*, 1994) within the MEGA 3.1 software (Kumar, S. *et al.*, 2004). As a result, synthetic oligonucleotides were designed to more precisely target the haplosporidian parasite DNA in PCR. These primers were designated Minch F1B and Minch R2B (Table 3.1) and amplify a 144 bp region of the SSU rRNA gene sequence. The Minch primers were used to amplify parasite DNA from histologically positive formalin fixed rock oyster samples containing the parasite described by Hine and Thorne (2002). The PCR products of four reactions were cloned and sequenced using the method outlined above.

The remaining SSU sequence of the rRNA gene was obtained by (i) using a new forward primer (FSSUF) for the sequence at the 5' end of the SSU rDNA paired with the Minch R2B primer and (ii) pairing the Minch F1B primer with the 16 s universal eukaryotic SSU rRNA gene primer for the sequence at the 3' end (Medlin, L., Elwood, H.J. and Sogin, M.L., 1988). The sequence generated in these reactions was compared to the overlapping sequence obtained from the HAP PCR reactions to ensure the correct organism and species was sequenced. Each of these PCR reactions was performed using the same method as for the HAP primers except the final concentration of $MgCl_2$ in the reactions was lowered to 1.5mM and the annealing temperature was 61°C for the FSSUF/Minch R2B reactions and 48°C for the Minch F1B/16 s b reactions. Extension temperatures were also raised to 72°C. The success of each PCR reaction was determined using the same method described for the PCR utilising HAP primers. The cloning and sequencing of these PCR products was

performed using the same method as the HAP primer reactions. The SSU rRNA gene sequence was obtained from the clones of three positive oysters each sequenced three times to give nine sequences. Each sequence was verified as belonging to a haplosporidian parasite using BLAST searches of the GenBank database.

3.2.6 In situ hybridisation

In order to identify the parasite in histological sections, the SSU rRNA gene sequence obtained was compared to other haplosporidian sequences and a region with high sequence variability was identified. This region was used to design a 30 bp oligonucleotide probe for use with *in situ* hybridisation. The probe was labeled with digoxigenin at the 5' end using a DIG Oligonucleotide Tailing Kit (Roche Diagnostics) according to the manufacturer's instructions. The probe was designated SSR 69 and its sequence is given in Table 3.1.

Labeling efficiency was assessed by spotting 1 µL serial aqueous dilutions of probe on a positively charged nylon membrane (Boehringer Mannheim). The DNA was fixed by baking at 120°C for 30 min, washed in buffer 1 (0.1 M Tris pH 7.5, 0.1 M NaCl, 2 mM $MgCl_2$, and 0.05% Triton X-100) for 2x5 min and incubated for 30 min in antibody (alkaline- phosphatase conjugated anti-digoxigenin Fab fragments; Roche Applied Science) diluted 1:5000 in buffer 1. After two 10 min washes in buffer 1, and 5min equilibrium in buffer 3 (0.1 M Tris pH 9.5, 0.1 M NaCl, and 50 mM $MgCl_2$), the reaction was developed overnight in BM purple (Roche Applied Science).

Formalin fixed oyster tissue was embedded in paraffin blocks. Unstained sections were cut 6 µm thick and placed on aminoalkylsilane-coated slides. Sections were

dewaxed with xylene and rehydrated in an ethanol series. They were then rinsed and immersed in pure water. The sections were digested in a Proteinase K solution (Proteinase K 0.5 g/ml, 0.5 mM Tris–HCl, pH 7.6) for 45 min and immersed in pure water at 4 °C (2x10 min). Post fixing was performed using 0.4% paraformaldehyde in x1 PBS pre cooled to 4°C for 20 min. Sections were then washed in pure water and incubated in a pre-hybridisation solution: 1mM Tris–HCl pH 7.4, 20 mM standard saline citrate (SSC), 0.1 mM EDTA, 5% (w/v) dextran sulfate, and 50%(v/v) formamide for 1 h at 42°C. Slides were covered in 50 µL of hybridisation solution: 1 µg labelled probe with 1 mM Tris–HCl pH 7.4, SSC 20 mM, 0.1mM EDTA, 5% dextran sulfate (w/v) and 50% (v/v) formamide, covered and denatured for 15 min at 95°C in an oven. The slides were then transferred to a 42°C oven and incubated overnight in a humid chamber. The hybridisation solution was drained off and the slides were rinsed and immersed in 40 mM SSC (2x20 min). This was followed by a 30 min incubation in a solution containing 0.1% SSC, 2 mM $MgCl_2$, 0.1% Triton X-100 at 42°C. Slides were then covered in Tris-buffered saline containing 3% bovine serum albumin and 0.1% Triton X-100 (TBSBT) for 5 min. Hybridisation of the probes was detected by incubating the sections in anti-digoxigenin alkaline-phosphatase conjugate prediluted 1:600 in TBSBT for 30 min. Sections were then washed in Tris-buffered saline and alkaline-phosphatase substrate buffer for 5 min each. Slides were then stained with BM purple (Roche Applied Science) overnight and finally counterstained with brazilin haematoxylin. Negative controls included PCR negative sections and incubation with an identical hybridisation mix but without any labeled PCR product.

3.2.7 Phylogenetic analysis

The SSU rRNA gene sequences employed in the phylogenetic analysis were obtained from GenBank. Sequences were aligned using CLUSTALW (Thompson, J.D., Higgins, D.G. and Gibson, T.J., 1994) in MEGA 3.1 software (Kumar, S., Tamura, K. and Nei, M., 2004). Alignments were visually checked and an opening gap penalty of 10 and a gap extension penalty of 3 for both pairwise and multiple alignments were found to be the most effective. Phylogenetic analysis was conducted using MEGA3.1 with the haplosporidian parasites of *Haliotis iris* (AF492442), *Ruditapes decussatus* (AY435093) and *Pandalus platyceros* (AY449715) as an out-group. Phylogenetic tree construction was performed using a maximum parsimony analysis with the close neighbour interchange (CNI) heuristic option with a search factor of 2 and random initial trees addition of 2000 replicates (Azevedo, C., Balseiro, P., Casal, G., Gestal, C., Aranguren, R., Stokes, N.A., Carnegie, R.B., Novoa, B., Burreson, E.M. and Figueras, A.J., 2006). Bootstrap values were also calculated over 300 replicates.

3.3 Results

A protozoan parasite identified as a haplosporidian was detected by PCR in rock oysters (*S. cucullata*) from northwest Australia using degenerate HAP primers. Of the sixty three oysters processed twenty nine were positive by *in-situ* hybridisation. The parasite measured 6 µm in diameter and was disseminated throughout the gonad follicles (Figure 3.4) and to a lesser extent the gills and digestive glands of the infected oyster. No parasites were observed in the connective (Leydig) tissue surrounding the follicles or infecting any other tissue in the oyster. The only parasite life stages observed were uninucleate naked cells (Figure 3.4) and rarely binucleate plasmodia. There appeared to be little in the way of a defensive response from the oyster to the parasite. Indeed, there appeared to be few haemocytes in the vicinity of the parasites or in the Leydig tissue surrounding the oyster reproductive tissue. No phagocytosis of parasite cells was observed.

The parasite was identified in haematoxylin-eosin stained sections using *in situ* hybridisation by designing a 30 bp oligonucleotide probe from the parasite's SSU rRNA gene. The oligonucleotide probe was labeled with dioxigenin at the 5' end and applied to unstained tissue sections in *in-situ* hybridisation.

The probe produced strong hybridisation signals with little background staining (Figure 3.5A). This signal was not reproduced in tissues processed from uninfected oysters or in sections where no labeled DNA probe was applied (Figure 3.5B).

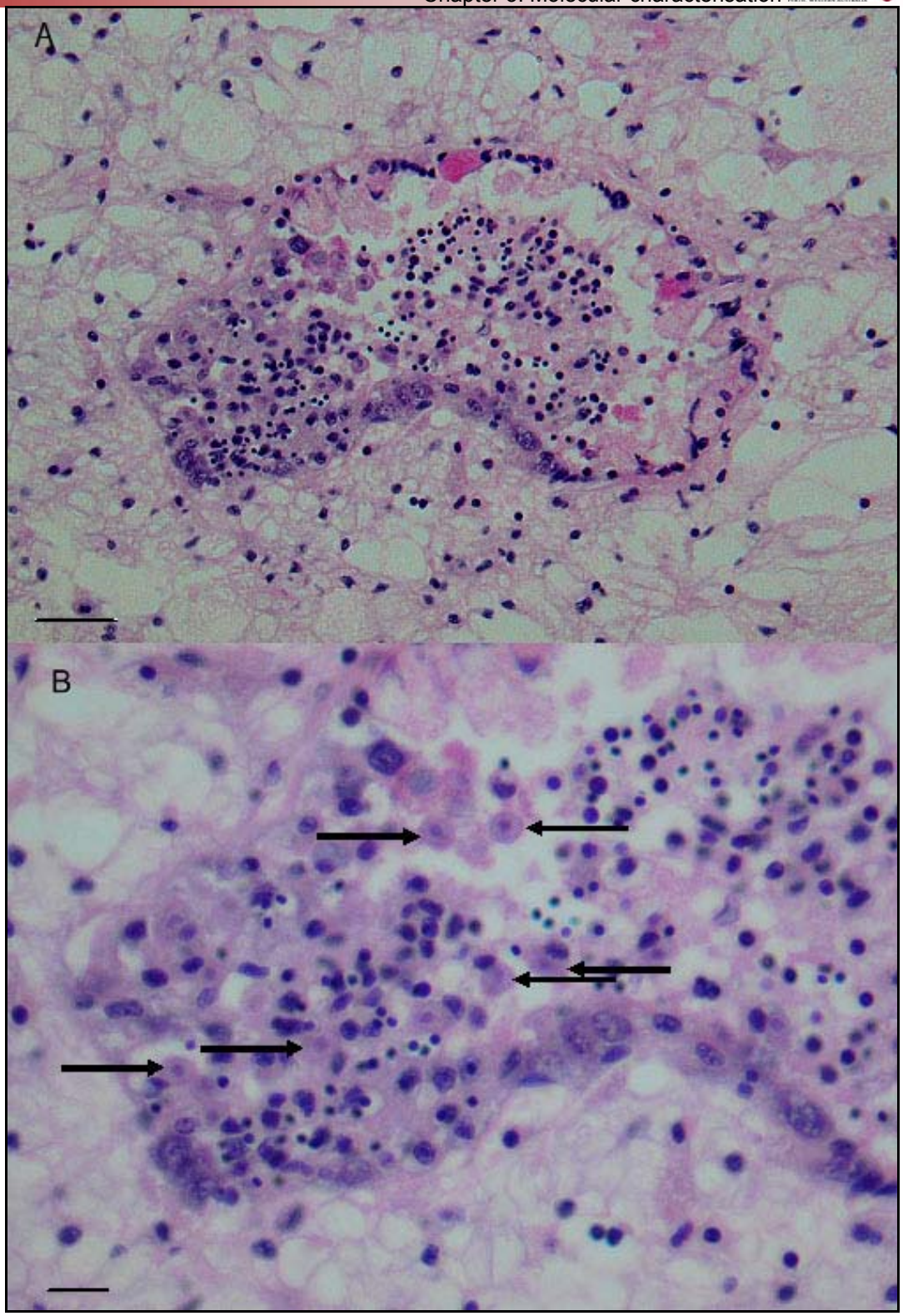


Figure 3.4 Rock oyster reproductive tissue containing haplosporidian parasites. (A) a hematoxylin-eosin stained section. Scale bar = 25 µm. (B) Higher magnification view of the same section. Parasites are identified with an arrow. Scale bar = 15 µm.

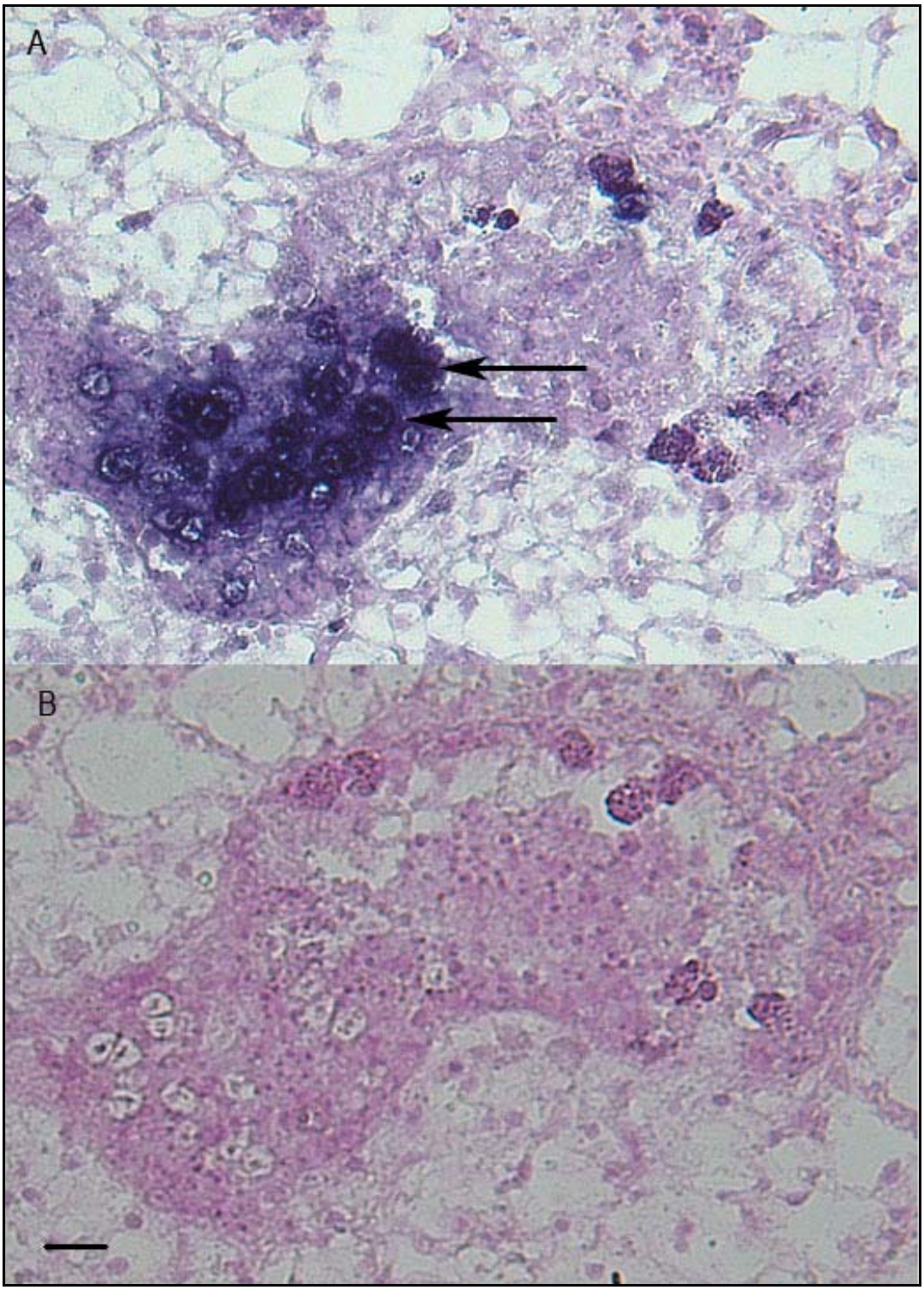


Figure 3.5 Haplosporidian parasites (arrows) identified in an *in-situ* hybridisation of rock oyster gonad follicles. (A) Parasites are identified by a darker colouration and are indicated by an arrow. (B) Negative control serial section from the same in-situ hybridisation. Sections are counterstained in a brazilin haematoxylin. Scale bar = 10 µm.

Table 3.2 The number of oysters sampled as well as the number of oysters diagnosed as positive from each of the sampling locations. Oysters were diagnosed by *in situ* hybridisation.

Location:	**n**	**positive**	**%**
Bluebell Island (D)	10	5	40
West bay (B)	22	10	41
Chartreude bay (A)	21	13	62
Crocus Island (C)	10	1	10
Total	**63**	**29**	**43**

The parasite was present at each of the four locations sampled (Table 3.2) although at varying prevalence. Chartreude Bay had the highest infection rate at 62% (13/21) while Crocus Island had the lowest at 1/10 (10%). None of the pearl oyster samples obtained from the Montebello Islands were positive (data not shown).

Spores were not detected so the parasite was not able to be identified using the established morphological criteria (Burreson, E.M., 2001). In order to carry out a phylogenetic analysis, additional primers were developed to sequence the remaining sections of the parasite's SSU rRNA gene (Table 3.1). The PCR products generated were cloned and sequenced. The 1682 bp SSU rRNA gene sequence was found to be unique, and consequently it was submitted to GenBank (Accession No. EF 165631). The sequence is also displayed in Appendix 1 along with the position of target sequences for the primers and probes used in the study.

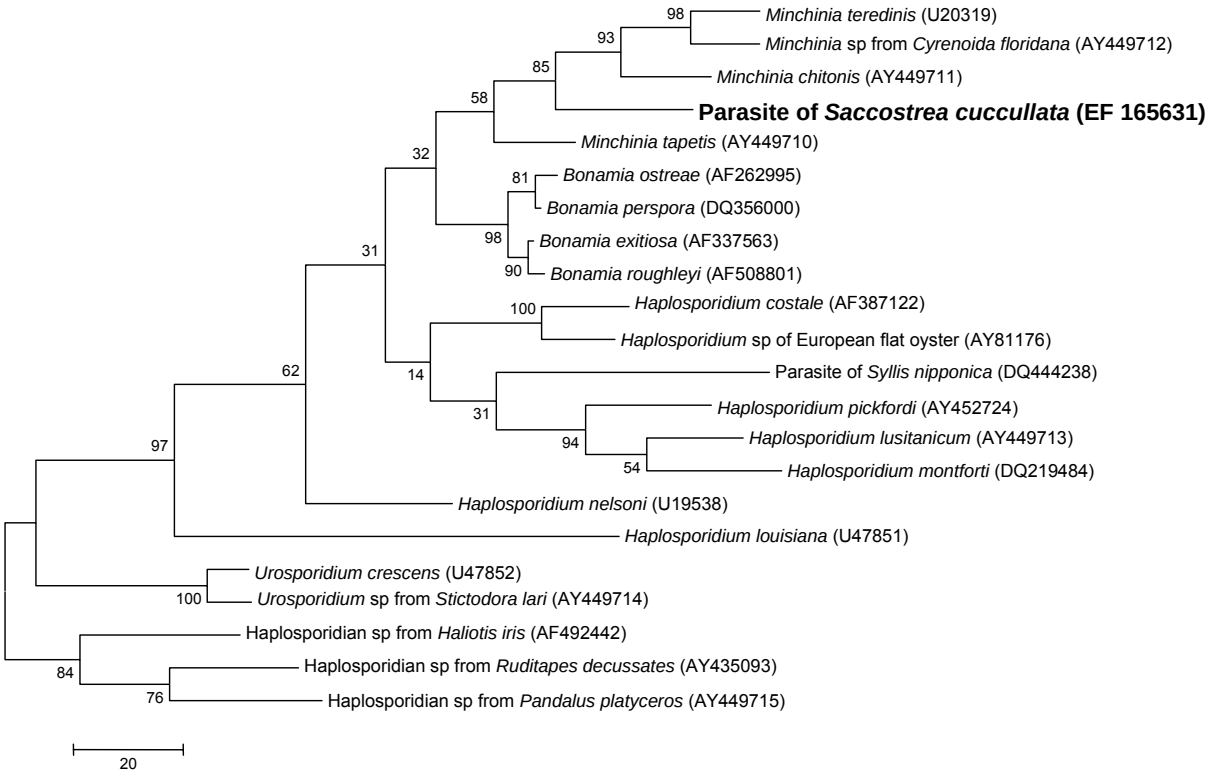


Figure 3.6 A maximum parsimony tree illustrating the relationships of the SSU rRNA gene sequences within phylum Haplosporidia including the rock oyster parasite. The analysis was conducted using 2218 aligned nucleotide positions with the previously reported basal haplosporidians as an outgroup. The analysis was supported by a 300 replicate bootstrap analysis. Numbers at nodes are percentages out of bootstrap analysis. Bar represents the equivalence between the distance and the number of changes.

The parasite SSU rRNA gene sequence obtained falls inside the genus *Minchinia* and is sister taxon to a clade composed of *M. chitonis*, *M. teredinis*, and *Minchinia* sp. Overall, bootstrap support for the monophyly of the genus *Minchinia* is at 58% (Figure 3.5). Monophyly for the genus *Bonamia* is strongly supported (98%) while support for the *Haplosporidium* excluding *H. nelsoni* and *Haplosporidium louisiana* is weakly supported at 14% (Figure 3.5).

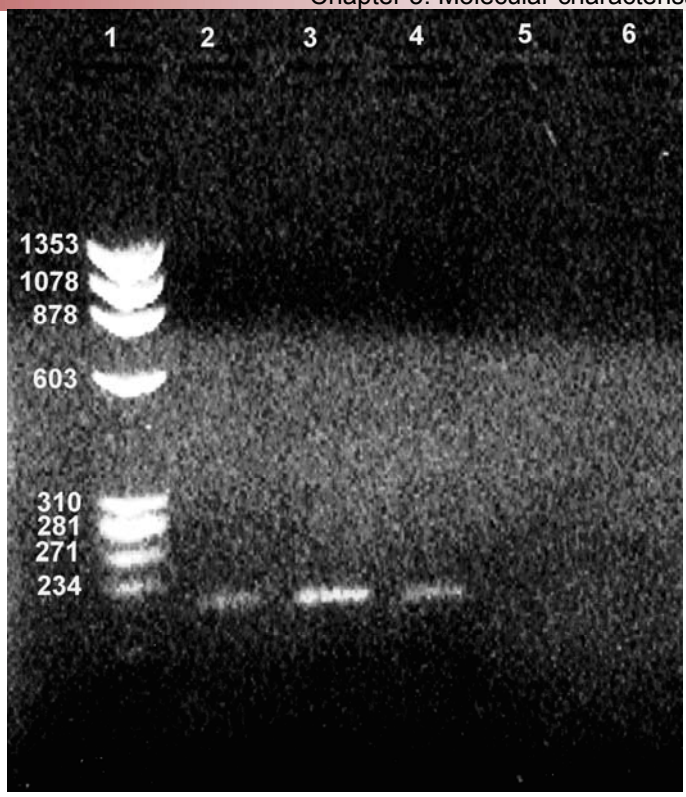


Figure 3.7 Agarose gel electrophoresis of the Minch PCR products from archived rock oyster tissues containing the haplosporidian parasite described by Hine and Thorne (2002). Samples were electrophoresed in a 2% agarose gel for 45 min at 46 milliamps. Lane 1: *Phi*X/*Hae*III molecular weight markers. The size of each marker in base pairs is indicated to the left. Lanes 2-4: PCR products from archived tissues. Lane 5: Negative control (no DNA).

The Minch F1B/R2B primers also were used to amplify a 144 bp section of formalin fixed archived samples of the parasite described by Hine and Thorne (2002). The Minch primers encompass a variable section of the SSU region of the parasite's rRNA gene. The Minch F1B/R2B reactions produced consistently positive results with no indication of contamination (Figure 3.6). When compared to the parasite detected in this study the sequences were within the levels expected for intraspecific variation.

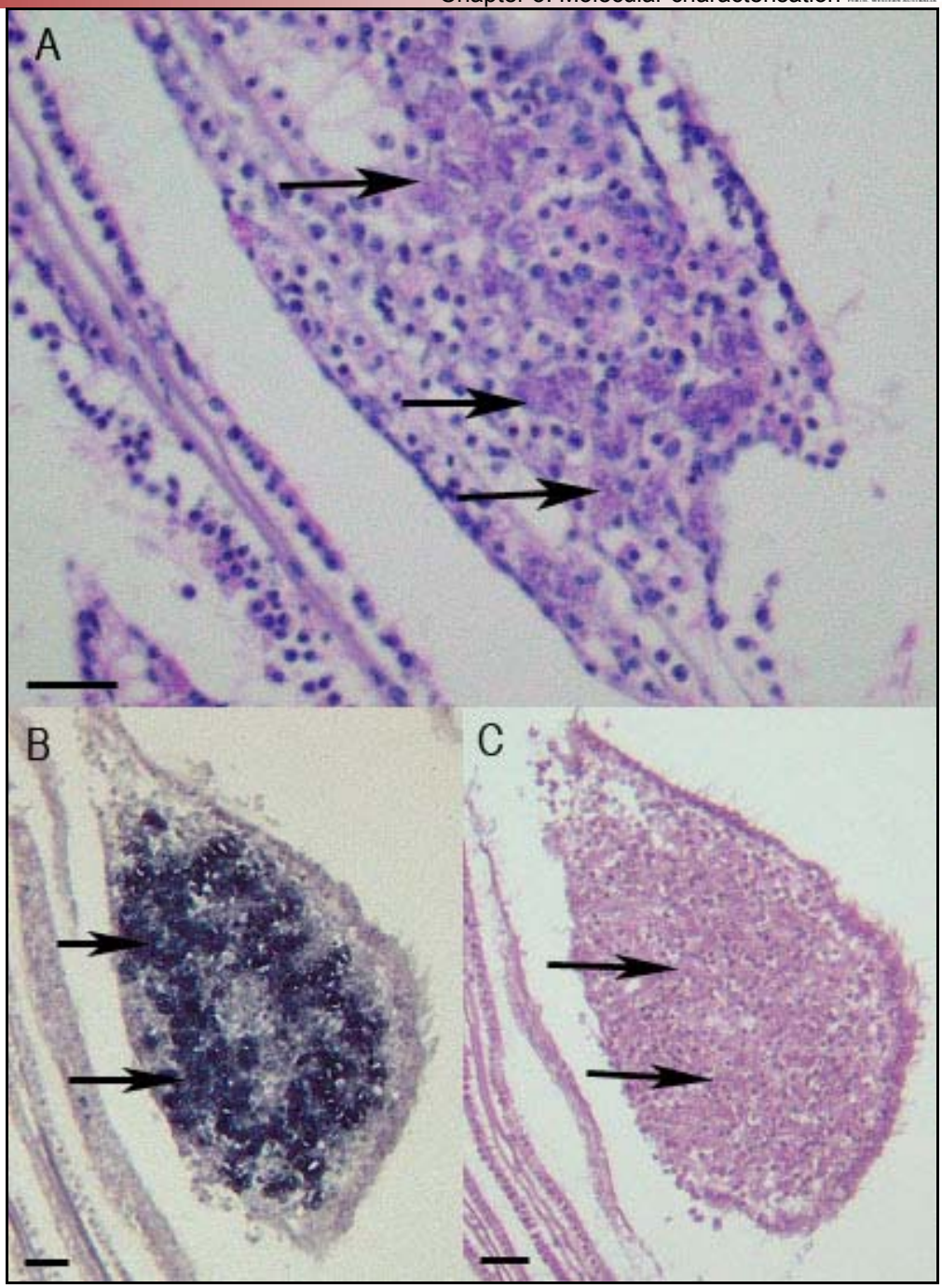


Figure 3.8 Serial sections of rock oyster gill tissue containing the parasite described by Hine and Thorne (2002). (A): A H/E stained section. Parasites are indicated with an arrow. (B): An *in situ* hybridisation assay containing the SSR 69 probe. (C): An *in situ* hybridisation assay with no probe in the hybridisation solution (negative control). Parasites are identified by a darker colouration. Sections are counterstained in a brazilin hematoxylin. All scale bars = 10 µm.

Overall, two sites within the 144 bp SSU sequence produced by the archived rock oyster parasites were different to that produced by the parasite detected in this study. At site 1489 the archived samples contained a T instead of a C, while at site 1459 the archived samples contained a G instead of an A (Appendix 1).

In order to ascertain whether the oligonucleotide probe reacted with the parasite described by Hine and Thorne (2002) an *in situ* hybridisation was performed on archived tissue used in that study. The *in situ* hybridisation produced strong hybridisation signals with little background staining (Figure 3.7).

3.4 Discussion

Haplosporidian parasites associated with oyster mortality have been infrequently detected on the north Western Australian coastline. However, the geographic isolation of the region and difficulties in locating the parasites by techniques such as traditional histology have hampered further research into the biology of these parasites (Hine, P.M. and Thorne, T., 2002).

The results obtained here demonstrate through PCR and *in situ* hybridisation the presence of a cryptic haplosporidian parasite in rock oysters. A phylogenetic analysis of the parasite indicates a *Minchinia* species between *Minchinia tapetis* and *Minchinia chitonis* (Figure 3.5). However, confirmation of this assessment, using spore ornamentation, is required. Spore ornamentation is the most accepted characteristic used to assess the taxonomic placement of haplosporidian parasites excepting the *Bonamia* species in which spores have not been detected (Burreson, E.M. and Ford, S.E., 2004). The most accepted morphological characteristic used to separate the *Minchinia* from the *Haplosporidium* is the presence of an epispore cytoplasmic extension (ECE) within the spore ornaments, while the *Haplosporidium* possess ornamentation consisting of spore wall material (Azevedo, C., Balseiro, P., Casal, G., Gestal, C., Aranguren, R., Stokes, N.A., Carnegie, R.B., Novoa, B., Burreson, E.M. and Figueras, A.J., 2006; Burreson, E.M. and Ford, S.E., 2004; Hine, P.M. and Thorne, T., 1998). Consequently, if the genus *Minchinia* is genuinely monophyletic, one would expect the spores of this parasite to possess ECEs. A monophyletic *Minchinia* genus is suggested with bootstrap support at 58% (Figure 3.5). The samples used in the present study were obtained from within the geographic range of a haplosporidian parasite described by Hine and Thorne (2002) from the same host.

The phylogenetic assessment of a *Minchinia* species is consistent with the parasite described by Hine and Thorne (2002) where a conclusive placement of the parasite in either *Minchinia* or *Haplosporidium* genera was not possible. An *in situ* hybridisation of archived rock oyster tissue infected with the parasite described by Hine and Thorne (2002) produced a positive reaction (Figure 3.7). A 144 bp sequence was obtained from archived formalin fixed tissues containing the parasite described by Hine and Thorne (2002). The sequence encompasses a variable region of the parasite's SSU rRNA gene. However, the sequence differed by only two base pairs from the SSU rRNA gene sequence obtained from the rock oyster parasite detected in this study. While only nine samples have tested positive to date, the parasite present in those samples appears unusual to other haplosporidians as the primary site of its detection is not connective tissue, gill or digestive gland epithelium but the gonad follicles of the host (Figures. 3.3 and 3.4). The association of the parasite with the host reproductive tissue may be subject to environmental or seasonal influence explaining the differences between the parasite detected in this study and the parasite described by Hine and Thorne (2002). Hine and Thorne (2002) noted the parasite had a higher prevalence of infection and more common sporogenesis in oysters with empty gonad follicles.

All of the oysters used in this study were leading towards spawning. Hine and Thorne (2002), who did find spores in their study, noted that the spore surface microtubules derived from the episporoplasm resemble similar microtubules in epispore cytoplasmic vacuoles of *Minchinia* in crabs except that the microtubules are unaligned and that only *Haplosporidium ascidiarum* from tunicates had similar unaligned microtubules. Thus, it is possible that *H. ascidiarum* may also be member of genus *Minchinia*.

The uni-nucleated naked cells identified as the origin of the *Minchinia* sequence in this study, appear similar to many *Bonamia* species. The *Minchinia* are a sister taxon to the *Bonamia* (Figure 3.5) and consequently the prevalence of uni-nucleated cells may be a result of the evolutionary relationship between these genera. Also, there appears to be little information in the literature on the life stages of many *Minchinia* species rather most authors appear to have preferred to concentrate on spore morphology. Speculatively, if the cryptic uni-nucleated stages detected in this study are a major part of the life-cycle of the *Minchinia* then this may explain this lack of information.

Chapter 4 : Detection of *Minchinia* sp. in rock oysters *Saccostrea cucullata* (Born, 1778) using DNA probes.

In this Chapter a number of molecular assays are characterized and assessed for sensitivity and specificity in an effort to validate the assays and describe their properties before they are used any further in the study.

4.1 Introduction

The effective control of diseases of aquatic animals requires access to diagnostic methods that are rapid, reliable, and highly sensitive. Histopathology has traditionally been used as the primary method for the diagnosis of shellfish diseases (Mialhe, E. *et al.*, 1995) since it can provide considerable information about the general health of a shellfish as well as the detection of a wide range of pathogens. However, histopathology requires professional training and many pathogens are difficult to detect by this method, if there are low numbers of parasites present within infected tissues. It can also be difficult to definitively diagnose infections based on parasite species morphology criteria.

DNA marker technology has several advantages over traditional techniques such as histopathology. DNA markers are extremely sensitive and are ideal for specifically recognizing target DNA sequences regardless of the life history stage present (Burreson, E.M. and Ford, S.E., 2004). The small ribosomal subunit (SSU) region of the rRNA gene has been widely targeted since it contains both conserved and variable regions interspaced within the sequence allowing the design of universal or specific markers. While personnel involved in the development of molecular markers require a considerable amount of professional training, the molecular assays once developed may be used by laboratory staff and technicians familiar with molecular diagnostic techniques.

Some caution needs to be applied to the use of molecular assays. Often it is not known whether an assay will detect all strains of a pathogen throughout its range or if the assay reacts with other pathogen species (Burreson, E.M. and Ford, S.E., 2004).

The assay should be sensitive enough to detect low levels of infection in positive samples. Consequently, qualification of the specificity and quantification of the sensitivity of a molecular assay is required and the assay needs to be validated against other techniques, such as histology, so that its reliability can be determined (Burreson, E.M. and Ford, S.E., 2004; Kleeman, S.N. *et al.*, 2002). The proper qualification and quantification of a molecular assay can take a considerable amount of time and can be an expensive process.

Two techniques utilising DNA marker technology are the polymerase chain reaction (PCR) and *in situ* hybridisation (ISH). Polymerase chain reaction allows high throughput of samples and enhanced sensitivity but can lead to false negatives (Burreson, E.M., 2000; Carnegie, R.B. *et al.*, 2003). Polymerase chain reaction also requires other visual confirmation methods so as to rule out the possibility of false positives from contamination. *In situ* hybridisation can determine the locality of the parasite within the host and allow subsequent morphological characterisation.

Several haplosporidian parasites have demonstrated the capacity to infect hosts other than those previously identified including *Haplosporidium nelsoni* and *Bonamia ostrea* (Burreson, E.M., Stokes, N.A. and Friedman, C.S., 2000b; OIE, 2006). This ability is a concern for the management of these parasites since many haplosporidians are cryptic and are likely to be missed in histology sections if they are not being specifically targeted.

The development of sensitive and specific PCR and ISH assays is required for a number of reasons. These include:

- Enable identification of the parasite's intermediate host despite what may be varying morphological features in an alternative stage in the parasites life-cycle.
- Understanding the distribution of the parasite within the host's tissues and the detection of any cryptic life stages in the host.
- To aid in determining the geographic distribution of the rock oyster parasite.
- Allow the sensitive detection of the rock oyster parasite in cross infection trials.
- To assess whether the rock oyster and pearl oyster haplosporidians are the same species or are present in the same tissues.

This chapter suggests a potential PCR assay for the *Minchinia* sp. detected and described in Chapter 3 and commences an assessment of its sensitivity and specificity. The 30 bp oligonucleotide ISH assay developed in Chapter 3 is also assessed. Both molecular methods are compared to histology. In addition, a polynucleotide ISH assay is also developed and assessed for specificity.

4.2 Methods and Materials

The sources of histologically positive bivalve sections infected with target parasites are indicated in Table 4.1. Ethanol stored and formalin fixed paraffin embedded samples of *Minchinia* sp. were obtained from the Montebello Islands (latitude -20.4' S longitude 115.53' E) as described previously in Chapter 2. Extracts of genomic oyster DNA infected with *Haplosporidium costale* and *H. nelsoni* were obtained from the Virginia Institute of Marine Science (VIMS).

Table 4.1 The sources of representative pathogens and host species used in the *in situ* hybridisation assays.

Parasite species*	Phylum	Host species	n	Sample obtained from	Location of infection@	Date of infection
Minchinia sp.	Haplosporidia	*Saccostrea cucullata*	20	As in Chapter 2	Montebello Islands.	2005
Minchinia teredinis	Haplosporidia	Shipworm *Teredo sp*	1	Virginia Institute of Marine Science.	Wachapreague Virginia USA	1985
Haplosporidium costale	Haplosporidia	*Crassostrea virginica*	1	Virginia Institute of Marine Science.	Wachapreague Virginia USA	2006
Haplosporidium nelsoni	Haplosporidia	*Crassostrea virginica*	1	Virginia Institute of Marine Science.	Machipongo River, Virginia USA.	2005
Bonamia roughleyi	Haplosporidia	*Saccostrea glomerata*	4	WA Department of Fisheries	Oyster Creek, Carnarvon	1995
Marteilia sp	Cercozoa	*Saccostrea cucullata*	4	WA Department of Fisheries	North West Shelf	1994
Rhynchodid-like ciliates	Ciliophora	*Pinctada maxima*	1	WA Department of Fisheries	Montebello Islands.	2001

* Representative samples were histologically positive. @ All samples were obtained from Western Australia unless otherwise stated.

4.2.1 DNA extraction.

Genomic DNA from infected rock oysters was extracted from approximately 5 mg of gill tissue using a Masterpure ™ DNA purification kit (Epicentre Technologies, Sydney) according to the manufacturer's protocol.

4.2.2 Amplification by Polymerase Chain Reaction.

The *Minchinia* sp. SSU rDNA gene sequence (Genbank accession number EF 165631) was aligned with the sequences of the haplosporidian parasites *Minchinia teredinis* (U20319), *Minchinia* sp from *Cyrenoida floridana* (AY449712), *Minchinia*

chitonis (AY449711), *Minchinia tapetis* (AY449710), *Bonamia ostreae* (AF262995), *Bonamia roughleyi* (AF508801), *Haplosporidium costale* (F387122), *Haplosporidium pickfordi* (AY452724), *Haplosporidium lusitanicum* (AY449713), *Haplosporidium nelsoni* (U19538), *Urosporidium crescens* (U47852) and host 18S sequences using the CLUSTALW algorithm within the MEGA 3.1 sequence analysis program (Kumar, S., Tamura, K. and Nei, M., 2004). The variable regions were assessed for sequences that appeared to be species specific. Sequences were then assessed for use as potential PCR primers using the software Genetool (Doubletwist). Once suitable priming regions were identified the potential primers were sent as queries to the Genbank database (http://www.ncbi.nlm.nih.gov/blast/) to determine if the primers would anneal to non target genes. Two oligonucleotides, designated SSF66 and SSR69 (Table 2) were selected and commercially synthesized (Geneworks, Sydney). The sequence of the SSR69 primer is the same as the *Minchinia* sp. ISH probe described earlier in Chapter 3. Genomic parasite and rock oyster DNA was subjected to polymerase chain reaction using the primers designated SSF66 and SSR69 (Table 4.2). Each PCR was performed in a total volume of 25 µL and contained 67 mM Tris-HCl, 16.6 mM $[NH_4]_2SO_4$, 0.45% Triton X-100, 0.2 mg/mL gelatin and 0.2 mM dNTP's, 1.5 mM of $MgCl_2$, 40 pmol of each primer, and 0.55 units of *Taq* polymerase and template DNA. Each of the reaction mixtures were subjected to an initial denaturation phase of 5 min at 94°C, then 35 amplification cycles, each cycle consisting of 30 sec of denaturation at 94°C, 30 sec of annealing at 62°C, 2 min of extension at 72°C and then a final 7 min extension at 72°C.

A 10 µL aliquot of each of the PCR products was then loaded on a 2% agarose gel and subjected to electrophoresis for 20 min at 95 volts. Detection of the PCR

products was performed using ethidium bromide staining. *Phi*X/*Hae* III (promega USA) markers were used to determine the size and concentration of the PCR products.

Primer specificity was tested in PCR reactions using genomic DNA extracted from oysters which were both positive and negative for *Minchinia* sp. by histopathology. *Haplosporidium nelsoni* and *Haplosporidium costale* DNA obtained from the Virginia Institute of Marine Science was also used to test PCR specificity. The infected oyster PCR reaction contained 50 ng genomic DNA isolated from the gill tissue of a histologically positive rock oyster collected from the Montebello Islands, Western Australia. To ensure the PCR assay did not cross react with host DNA a 500 ng aliquot of genomic oyster DNA isolated from the gill tissue of an uninfected oyster was also tested.

Table 4.2 Polymerase Chain Reaction primer sequences employed in Chapter 4.

Primer	**Sequence (5' -3')**	**Position**	**Use**	**Reference**
SSF 66	ccg cgc gat gcc cag ccg tat	551	PCR	Present Chapter
SSR 69	agc cca aaa cca aca aaa cgt cca cat gcg	750	PCR and *in situ* probe	Chapter 3
MinchF1B	ctc gcg ggc tca gct t	1295	Generate *in situ* probe	Chapter 3
MinchR2B	ggc gct ttg cag att ccc ca	1439	Generate *in situ* probe	Chapter 3

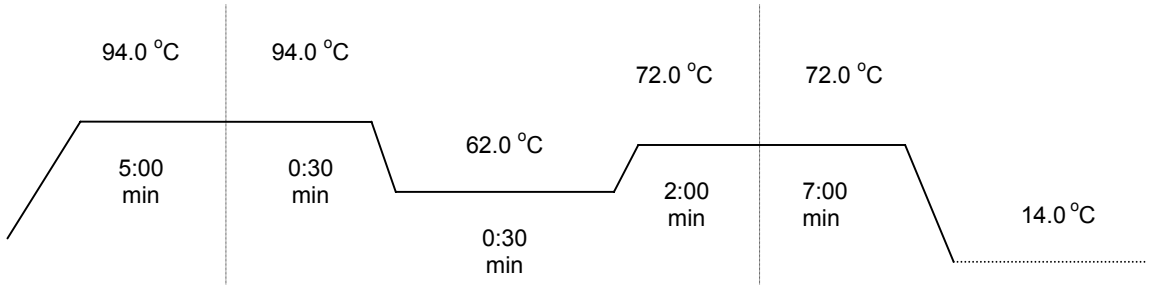


Figure 4.1 Polymerase Chain Reaction (PCR) conditions used for the PCR assay with the SSF66 and SSR69. Each of the PCR reactions was subject to 35 cycles.

4.2.3 Generation of polynucleotide probe for ISH.

The first ISH assay utilised a 144 bp polynucleotide probe which was generated by PCR. Genomic parasite and rock oyster DNA was subjected to polymerase chain reaction using the primers designated Minch F1B and Minch R2B (Table 4.2). Each PCR was performed using reaction mixtures that had a total volume of 50 µL and contained 67 mM Tris-HCl, 16.6 mM $[NH_4]_2SO_4$, 0.45% Triton X-100, 0.2 mg/mL gelatin and 0.2 mM dNTP's, 2 mM of $MgCl_2$, 40 pmol of each primer, and 0.55 units of *Taq* polymerase and template DNA. Each of the reaction mixtures were subjected to an initial denaturation phase of 5 min at 94°C, then 35 amplification cycles, each cycle consisting of 30 sec of denaturation at 94°C, 30 sec of annealing at 48° C , 2 min of extension at 68°C and then a final 7 min extension at 68°C (Figure 4.2). The successful amplification of target DNA by each PCR reaction was determined using the method outlined above.

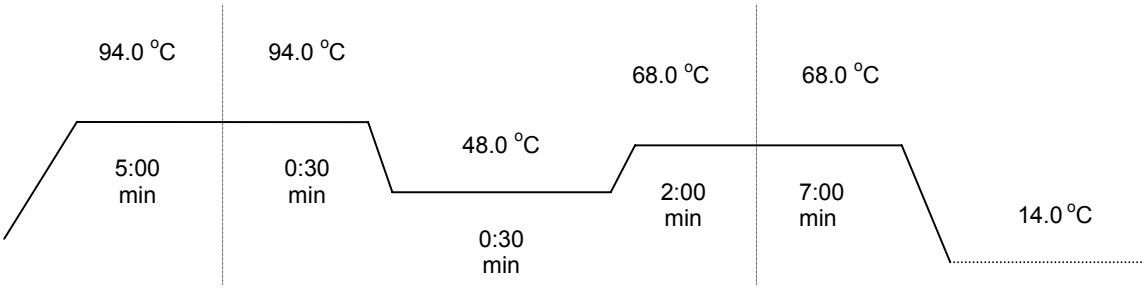


Figure 4.2 Polymerase Chain Reaction (PCR) conditions used for DNA amplification of the SSU rRNA gene utilised in the study. Each of the PCR reactions was subjected to 35 cycles.

4.2.4 In-situ hybridisation

A 144 bp polynucleotide probe obtained by PCR using the primers Minch F1B and Minch R2B (Table 4.2) was purified with a QiAquick PCR purification kit (Qiagen) and labeled using a nick-translation mix (Boehringer-Mannheim), both according to manufacturer's instructions. The second ISH assay used a 30 bp synthetic oligonucleotide (SSR69*; Table 4.2) and was labeled using a DIG Oligonucleotide Tailing Kit (Roche diagnostics).

The presence and location of each parasite species was confirmed using hematoxylin-eosin (H&E) stained serial sections. The *in situ* hybridisation procedure was performed as in Chapter 3. The assay was optimised by varying the Proteinase K incubation time and the concentration of the DIG-labeled probe. The optimal Proteinase K incubation time was found to be 15 min (at 50 µg/ml) while the optimal concentration of DIG labeled probe was found to be 2 ng/ml of hybridisation mix. Negative controls included histologically uninfected sections and incubation with an

identical hybridisation mix but containing an irrelevant oligonucleotide probe. Parasite identity and location were confirmed in adjacent sections stained with H&E.

4.2.5 Minchinia sp. diagnosis

In order to compare the diagnostic capabilities of the *Minchinia* sp. primers and the oligonucleotide ISH assay against the established techniques of histological examination, 56 oysters were tested using all three techniques to determine the presence of the *Minchinia* sp. in each individual. Histological examinations using H&E stained sections were undertaken first, with sections examined for approximately five minutes each. The PCR assay was performed next followed by ISH. The oligonucleotide ISH assay was performed using serial sections cut adjacent to the sections stained for H&E examination. Since the rate of detection in histology depends in part on the ability of the operator, a retrospective examination of the H&E stained serial sections was performed excepting sections that were negative by all three tests. The use of serial sections between the ISH and H&E stained sections meant the retrospective histological examination could be performed in less than 5 minutes since the location of the parasites could be identified within the positive ISH section. Each oyster was examined only once by the PCR and ISH assays and only a single H&E section was taken from each oyster.

The results of the comparison were evaluated using standard epidemiological methods (OIE, 2006) for sensitivity (proportion of oysters with *Minchinia* sp. that test positive – proportion of true positives), specificity (proportion of oysters without *Minchinia* sp, that test negative – proportion of true negatives), positive predictive

value (PPV; the probability that an oyster returning a positive test is actually positive) and negative predictive value (NPV; the probability that an oyster that returns a negative test actually does not have *Minchinia* sp). The results from the molecular methods were assessed against each of the histological examinations. A chi-squared test was performed using SPSS for Windows (version 13.0) and compared the number of positives generated by each diagnostic test. The results for each diagnostic test were treated as either positive or negative regardless of the level of infection.

4.3 Results

4.3.1 Polymerase Chain Reaction

The PCR primer pair SSF66/SSR69 amplified a 220 bp region of the *Minchinia* sp. small subunit rRNA gene (Figure 4.3). The assay did not amplify the SSU sequences from *H. nelsoni* or *H. costale* infected oyster genomic DNA (Figure 4.3). The primers did not produce a product from 500 ng of genomic DNA from an oyster not infected with *Minchinia* DNA (Figure 4.3).

The product was easily detectable after PCR amplification using 10 fg of *Minchinia* sp. amplified DNA (Figure 4.4). The primers amplified the *Minchinia* sp. SSU rDNA from 50 ng of genomic DNA from an oyster histologically positive for the *Minchinia* sp. (Figure 4.4).

It was evident that increasing amounts of host DNA hindered the PCR when concentrations greater than 250 ng were added to the reaction (Figure 4.5). The PCR failed when 300 ng was added to the reaction.

4.3.2 In-situ hybridisation: DNA probe specificity

In order to assess the specificity of the *in situ* hybridisation assays using two different SSU rDNA probes, paraffin embedded samples of oysters infected with a variety of parasites from the phyla Haplosporidia and Ciliophora, as well as a paramyxian, were subjected to *in situ* hybridisation. Parasite identity and location were confirmed in adjacent sections after staining with Hematoxylin-eosin (H&E; Figure 4.6).

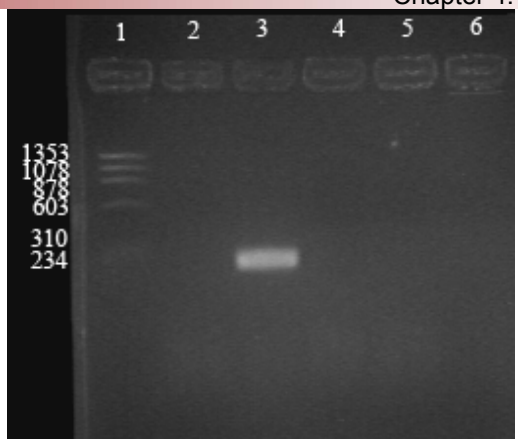


Figure 4.3 Agarose gel electrophoresis of the SSF66/SSR69 PCR products demonstrating specificity for *Minchinia* sp. Samples were electrophoresed in a 2% agarose gel for 45 min at 90V. Lane 1: *Ph*iX/*Hae*III molecular weight markers. The size of each marker in base pairs is indicated to the left. Lane 2: No DNA negative control. Lane 3: Positive *Minchinia* sp. infected rock oyster sample. Lane 4:Uninfected rock oyster. Lane 5: *Haplosporidium nelsoni* sample. Lane 6: *Haplosporidium costale* sample.

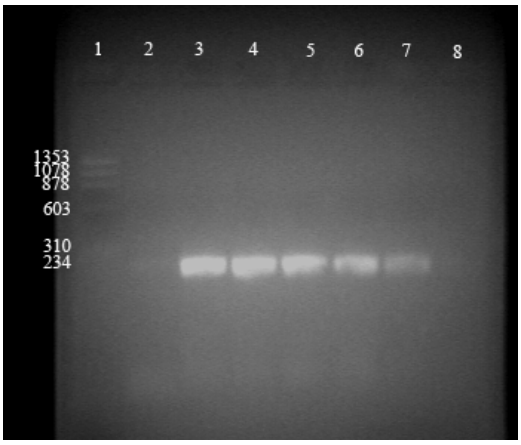


Figure 4.4 Agarose gel electrophoresis of the SSF66/SSR69 PCR products with varying amounts of amplified *Minchinia* sp. Samples were electrophoresed in a 2% agarose gel for 45 min at 90V. Lane 1: *Ph*iX/*Hae*III molecular weight markers. The size of each marker in base pairs is indicated to the left. Lane 2: No DNA negative control. Lane 3: 1 ng of template DNA. Lane 4: 100 pg. Lane 5: 10 pg. Lane 6: 1 pg. Lane 7 10 fg. Lane 8 1 fg

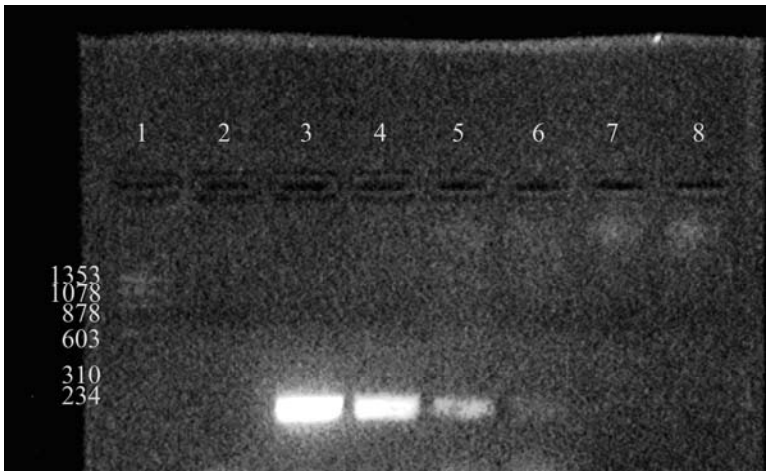


Figure 4.5 Agarose gel electrophoresis of the SSF66/SSR69 PCR products with of 1 pg of amplified *Minchinia* sp. DNA and varying amounts of host DNA. Samples were electrophoresed in a 2% agarose gel for 45 min at 90V. Lane 1: *Ph*iX/*Hae*III molecular weight markers. The size of each marker in base pairs is indicated to the left. Lane 2: No DNA negative control. Lane 3: Positive control. Lane 4: 200 ng of host DNA. Lane 5: 250 ng. Lane 6: 300 ng. Lane 7: 400 ng. Lane 8 500 ng.

Both the ISH probes were found to hybridise to all presporulating and sporulating stages of the *Minchinia* sp. in rock oyster sections (*Saccostrea cucullata*; Figure 4.6). The polynucleotide probe also detected intracellular *Bonamia roughleyi* infecting the digestive gland of a Sydney rock oyster (Figure 4.7) and produced positive results from the plasmodial stages of all of the other haplosporidian parasites tested (Figures 4.7 and 4.8) including *Haplosporidium nelsoni* (Figure 4.7) *and H. costale* infecting the digestive gland of an Eastern Oyster (*Crassostrea virginica*; Figure 4.8) and *Minchinia teredinis* from the gills of the shipworm *Teredo* sp (Figure 4.8). Both probes produced little background staining and did not reproduce a signal in tissues processed from uninfected oysters or in other negative control material where an irrelevant oligonucleotide probe was applied (Figures 4.5 to 4.8). Both probes failed to detect the paramyxian parasite (*Marteilia* sp) or the ciliate (data not shown).

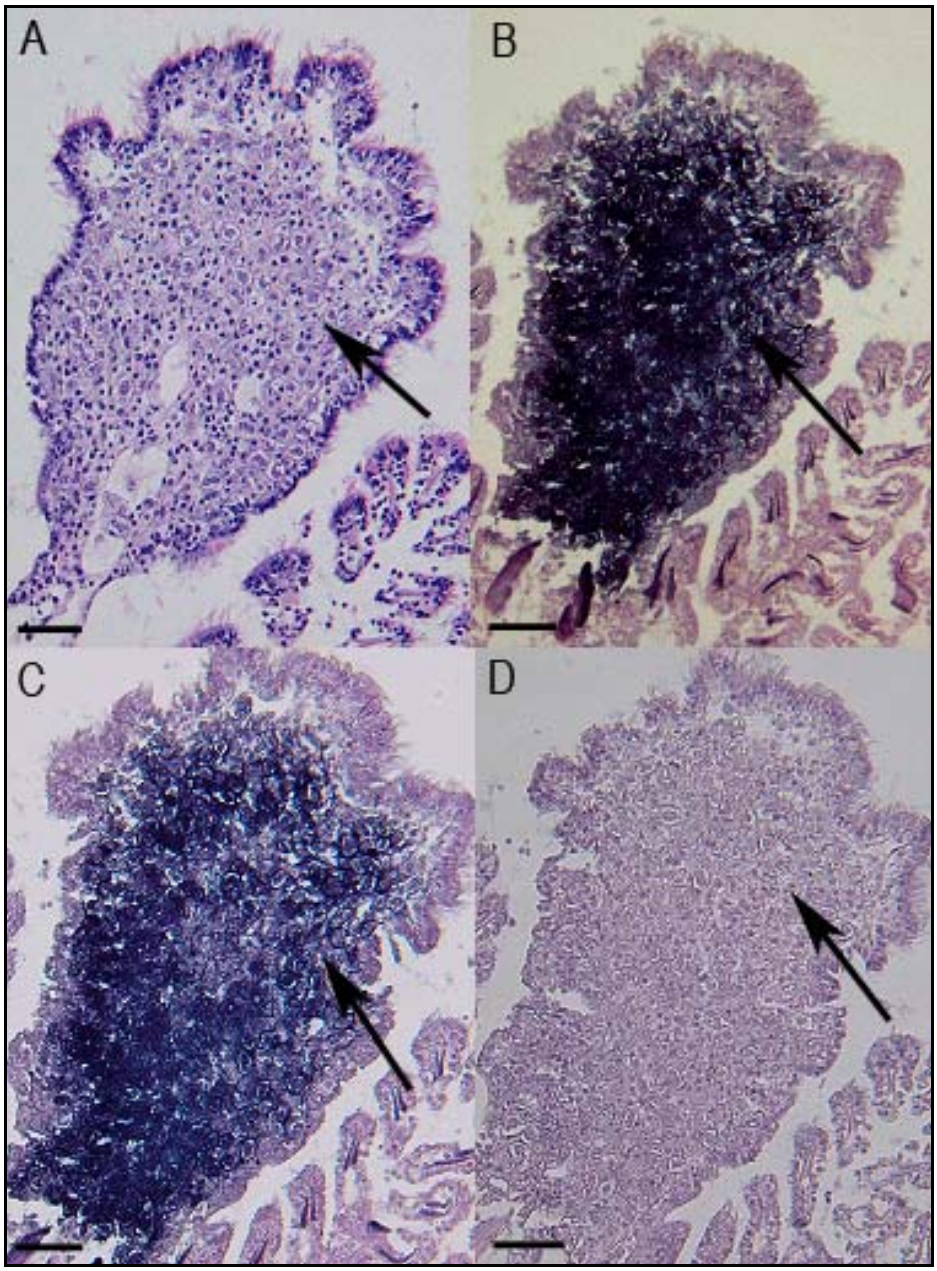


Figure 4.6 *In-situ* hybridisation both the SSR69 and polynucleotide ISH probes on a gill lesion from the rock oyster parasite described by Hine and Thorne (2002). (A): Hematoxylin-eosin stained section containing *Minchinia* sp. in rock oyster gill tissue. (B): Serial section used in an *in situ* hybridisation with the polynucleotide probe. Parasites are identified by a darker colouration. (C): Serial section used in an *in situ* hybridisation with the oligonucleotide probe. Sections are counterstained in a brazilin hematoxylin. (D): Negative control *in situ* hybridisation with an irrelevant probe. Scale bar is equal to 15 µm and applies to each picture. Example parasites are indicated by the arrow.

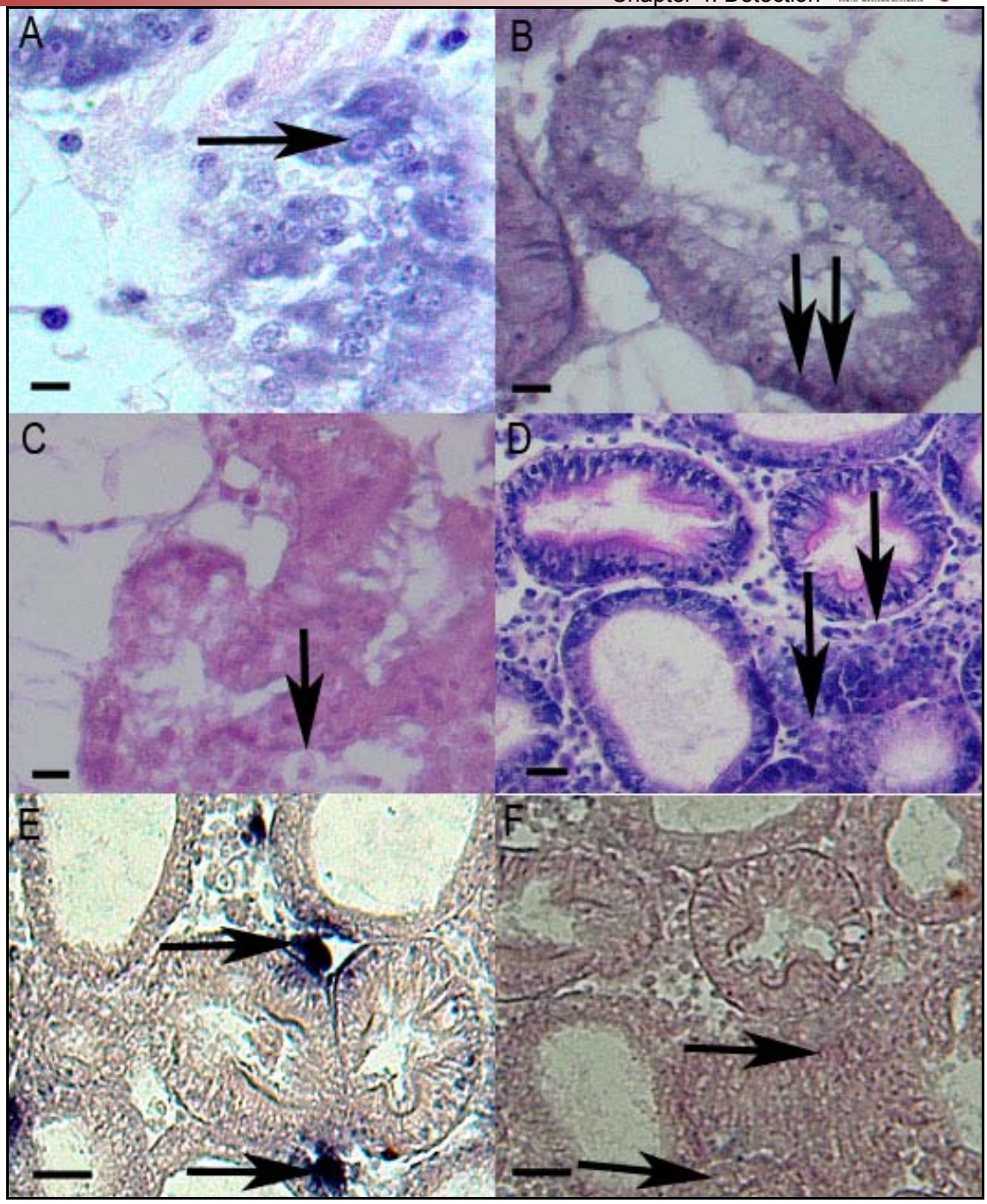


Figure 4.7 Sections of various haplosporidian parasites used to test the specificity of the ISH probes. (A): Hematoxylin-eosin stained section containing *Bonamia roughleyi* in a Sydney rock oyster digestive diverticula. Scale bar = 5 µm (B): Polynucleotide in situ hybridisation from the same region. Parasites are identified by a darker colouration. Scale bar = 15 µm. (C): Oligonucleotide ISH hybridisation also from the same region. Scale bar = 5 µm. Sections are counterstained in a brazilin hematoxylin. (D): H/E of *Haplosporidium nelsoni* plasmodia in an Eastern Oyster (*Crassostrea virginica*) digestive gland. Scale bar = 5 µm (E): Serial section used in an ISH with the polynucleotide probe. Parasites are identified by a darker colouration. Scale bar = 15 µm. (F): Serial section used in an *in situ* hybridisation with the oligonucleotide probe. Scale bar = 15 µm.

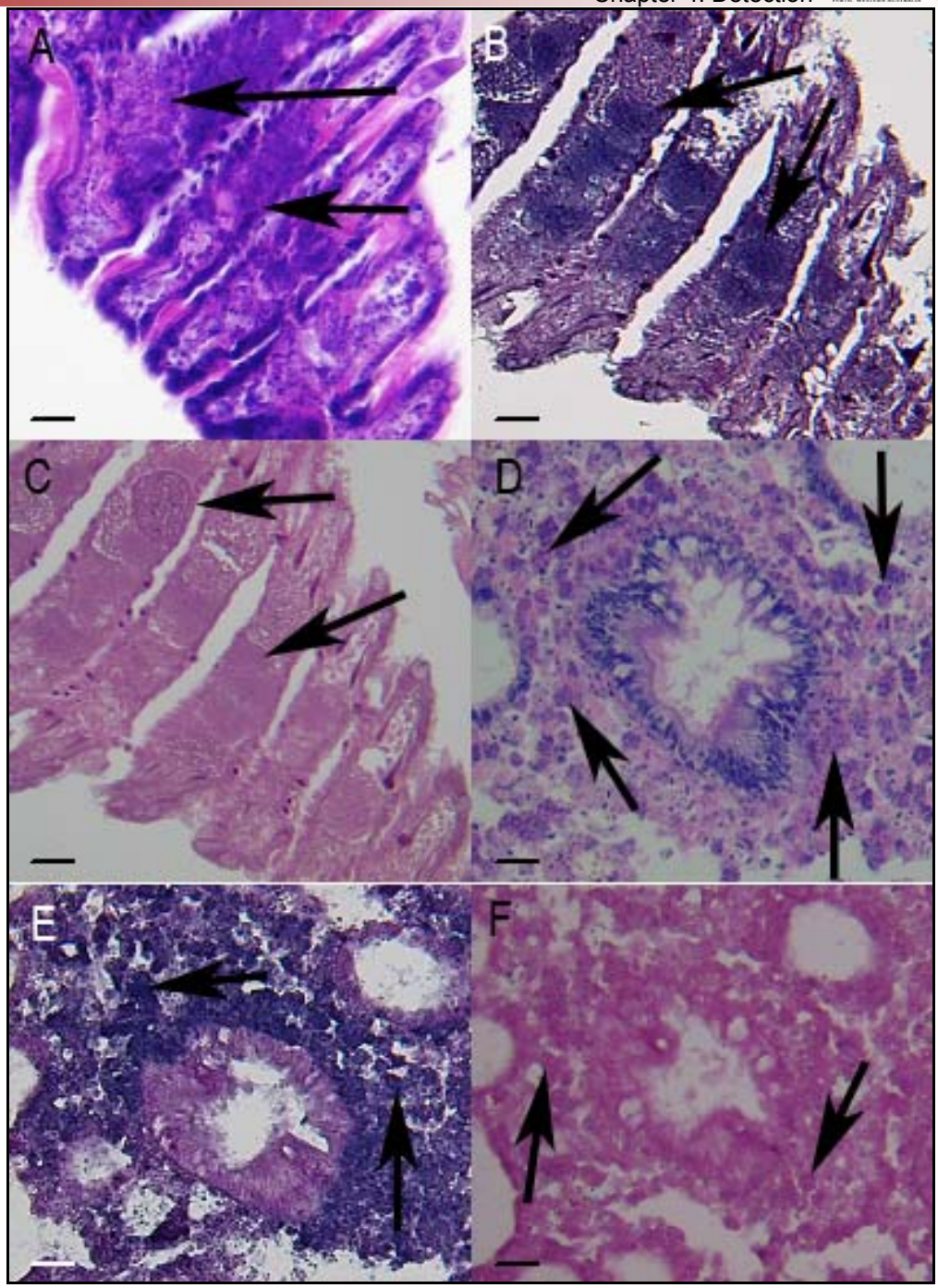


Figure 4.8 Sections of various haplosporidian parasites used to test the specificity of the ISH probes. (A): Hematoxylin-eosin stained section containing *Minchinia teredinis* in *Teredo* sp. (B): Serial section used in an ISH with the polynucleotide probe. Scale bar = 15 µm. Parasites are identified by a darker colouration. (C): Serial section used in an ISH with the oligonucleotide probe. Scale bar = 15 µm. (D): H/E stained section containing *Haplosporidium costale* plasmodia in an Eastern Oyster (*Crassostrea virginica*) digestive gland. Scale bar = 10 µm. (E): Serial section used in an ISH with the polynucleotide probe. Scale bar = 10 µm. (F): Serial section used in an ISH with the oligonucleotide probe. Scale bar = 10 µm.

4.3.3 Sensitivity

Both probes recognised all presporulating *Minchinia* sp. stages in repeated assays including uninucleate and binucleate stages (Figure 4.6). Plasmodia also produced strong positive results along with immature sporonts. The assay did not detect *Minchinia* sp. spores.

4.3.4 Diagnosis comparison.

Infection by *Minchinia* sp. was detected by histological examination (an initial examination and a retrospective examination) PCR and *in situ* hybridisation with the oligonucleotide probe. The parasites were generally present at low levels of infection making them difficult to detect by histological examination alone. Parasites were detected in the reproductive follicles, digestive gland and gills of the host. The initial histological examination detected 14 infected oysters among the 56 oysters examined while PCR amplification detected 26 infected oysters (Table 4.3). *In-situ* hybridisation detected a total of 29 infected oysters (Table 4.3).

Initial histological examination diagnosed thirteen oysters as uninfected that were described as infected by PCR (Oysters 7, 8, 11, 14, 17, 18, 20, 35, 37,43, 47, 53, and 56 ; Table 4.3). These oysters were diagnosed as a light infection by *in situ* hybridisation. Once PCR and ISH assays were competed, a retrospective histological examination was performed. The retrospective histological examination detected parasites in eight of these oysters (oysters: 14, 17, 18, 20, 33, 37, and 47) previously described as uninfected. The intensity of infection determined by histological examination was often lower than that determined by *in situ* hybridisation. PCR

diagnosed 3 oysters as negative that were described as a light infection by histological examination and *in situ* hybridisation (oyster 10, 17 and 43; Table 4.3).

Table 4.3 Comparison of histology, *in-situ* hybridisation (ISH) and PCR for detecting *Minchinia* sp. in 56 rock oysters.

Oyster number	Histology	PCR	ISH	Retrospective histology	All tests combined
1	heavy	positive	heavy	heavy	heavy
2	medium	positive	heavy	medium	heavy
3	negative	negative	negative		negative
4	light	positive	light	light	light
5	medium	positive	medium	medium	medium
6	negative	negative	negative		negative
7	**negative**	positive	light	negative	light
8	**negative**	positive	light	negative	light
9	negative	negative	negative		negative
10	light	**negative**	light	light	light
11	**negative**	positive	light	negative	light
12	negative	negative	negative		negative
13	negative	negative	negative		negative
14	**negative**	positive	light	very light*	light
15	negative	negative	negative		negative
16	negative	negative	negative		negative
17	**negative**	**negative**	light	very light*	light
18	**negative**	positive	light	very light*	light
19	negative	negative	negative		negative
20	**negative**	positive	light	very light*	light
21	light	positive	medium	light	medium
22	negative	negative	negative		negative
23	light	positive	light	light	light
24	negative	negative	negative		negative
25	light	positive	light	light	light
26	**negative**	positive	light	negative	light
27	negative	negative	negative		negative
28	**negative**	positive	light	very light*	light
29	light	positive	light	light	light
30	negative	negative	negative		negative
31	negative	negative	negative		negative
32	negative	negative	negative		negative
33	**negative**	positive	light	very light*	light
34	light	positive	light	light	light
35	negative	negative	negative		negative
36	medium	positive	medium	medium	medium
37	**negative**	positive	light	very light*	light
38	negative	negative	negative		negative
39	negative	negative	negative		negative
40	negative	negative	negative		negative
41	negative	negative	negative		negative
42	light	positive	medium	light	light
43	**negative**	**negative**	light	very light*	light
44	Negative	negative	negative		negative
45	light	positive	medium	light	light
46	negative	negative	negative		negative
47	**negative**	positive	light	very light*	light
48	negative	negative	negative		negative
49	negative	negative	negative		negative
50	light	positive	light	light	light
51	negative	negative	negative		negative
52	negative	negative	negative		negative
53	**negative**	positive	light	negative	light
54	negative	negative	negative		negative
55	negative	negative	negative		negative
56	**negative**	positive	very Light	negative	very light
Diagnosed positive (n)	14	26	29	23	29
Apparent prevalence	25%	46 %	52%	41 %	52 %
False negatives[#]	26.8 %	5.4 %	0 %	10.7 %	0 %
Chi-square statistic	14.0	.286	0.71	1.786	
df	1	1	1	1	
Significance	.000	.593	.789	.181	

Heavy is defined as more than 20 parasites per x20 field of view, medium is between 10-19 parasites per x20 field of view light is between 4-9 parasites per x20 field of view while very light refers between 1-3 parasites per x20 field of view. # False negatives defined relative to the all tests combined category. * Represents a change between the initial histological examination and the retrospective histological examination. Diagnoses in bold are in disagreement with the alternative tests.

The traditional diagnostic method provided an apparent *Minchinia* sp. prevalence of 25% (Table 4.4). In comparison, ISH provided the highest apparent prevalence of 52% with the highest sensitivity of 100% and an NPV of 100%. However, the specificity and PPV of ISH was relatively low (64.2% and 48.2%; Table 4.4) compared to histology because ISH allowed visualisation of low intensity *Minchinia* infections in 15 oysters which were negative by histology. A chi-squared test detected a significantly different result between the ISH assay and histology but a significantly different result could not be obtained compared to PCR. The retrospective histological examination, performed with the benefit of a serial ISH section, diagnosed 23 positive oysters at 41% prevalence. A chi-squared test did not detect a significantly different result between the retrospective examination and the PCR and ISH assays.

Polymerase chain reaction had a sensitivity of 92.8% and this method recorded an apparent prevalence of 46% (Table 4.4). The NPV of PCR was relatively low at 75% since this method failed to detect three low intensity *Minchinia* sp infections in three oysters, which were positive in ISH and retrospective histological examination. Specificity and PPV were again relatively low (69% and 50%) compared to histology because PCR obtained positive results from 12 oysters which were negative by histology (but positive by ISH). A chi-squared test detected a significantly different result between the PCR assay compared to the initial histological examination.

When histology was compared to the pooled PCR and ISH results, the molecular methods provided an apparent *Minchinia* sp. prevalence of 52% (Table 4.5). The sensitivity of histology compared to the pooled molecular methods was relatively low

at 48 % with an NPV of 64%. This was because of 15 false negative results when compared to the molecular methods. Specificity and PPV were both 100% for histology (Table 4.5).

Table 4.4 Evaluation of each of the diagnostic assays; PCR and ISH relative to histology.

	PCR	**ISH**	**Histology**
Sensitivity	92.8%	100%	100%
Specificity	69.0%	64.2%	100%
PPV*	50%	48.2%	100%
NPV*	75%	100%	100%
Apparent prevalence	46%	52%	25%
True prevalence	25%	25%	25%

*PPV is the positive predictive value, NPV is the negative predictive value.

Table 4.5 Evaluation of each of the diagnostic assays; Histology compared to ISH and PCR.

	Histology	**PCR/ISH**
Sensitivity	48%	100%
Specificity	100%	100%
PPV*	100%	100%
NPV*	64%	100%
Apparent prevalence	25%	52%
True prevalence	52%	52%

*PPV is the positive predictive value, NPV is the negative predictive value.

4.4 Discussion

These results indicate that both the PCR assay developed in this study and the *in-situ* hybridisation assay developed in Chapter 3 were capable of detecting *Minchinia* sp. in rock oysters. The comparison of diagnostic techniques suggested the ISH assay was the most sensitive followed by PCR (Tables 4.3 and 4.4).

4.4.1 Specificity and sensitivity of the PCR assay.

The SSF66/SSR69 PCR assay targeted variable sections within the SSU region of the parasite's rRNA gene. The assay recognised only the *Minchinia* sp. infected rock oyster and did not detect either *Haplosporidium nelsoni* or *Haplosporidium costale* DNA (Figure 4.3). Further testing is required using closely related *Minchinia* species such as *M. tapetis* and *M. teredinis* in order to confirm the PCR assay doesn't cross react with these species. All of the negative control material including uninfected oysters and irrelevant probe controls were negative.

The assay did appear to suffer from the focal or patchy distribution of the parasite in the host. One oyster produced a negative result by PCR which was subsequently found to be positive by the initial histological examination (oyster 10; Table 4.3) while two oysters were found to be very lightly infected in the retrospective histological examination (oysters 17 and 43; Table 4.3). All three of these oysters were diagnosed as infected by the oligonucleotide ISH assay. These results suggest that DNA was extracted from tissues of the host that did not contain sufficient numbers of parasites to produce a positive PCR result and that section-based methods are potentially more accurate because more tissue types are examined. The parasite appears to have a localised distribution in lightly infected oysters, and hence it may be

difficult to detect early or very light infections using PCR. A similar result was also obtained by Diggles *et al* (2003) in their comparison of diagnostic techniques for *Bonamia exitiosus* from flat oysters (*Ostrea chilensis*) in New Zealand.

The amount of host DNA in the reproductive tissue can be substantial and needs to be greatly diluted to avoid inhibition of the PCR reaction. A small quantity of target DNA in a very high amount of host DNA can result in a marked decrease in PCR sensitivity, perhaps because of decreased potential for primer template binding (Zimmermann, K. *et al.*, 1994). The assay was successful in detecting 10 fg of template DNA in 250 ng of host DNA (Figure 4.5).

While PCR doesn't allow quantification of infection intensity, it did detect low intensity infections in the majority of cases when the results of the tests were compared for each oyster (Table 4.3). Histological examination did not detect fifteen oysters described as infected by PCR and/or *in-situ* hybridisation. Subsequent re-examination of these oysters resulted in an additional nine infections being detected (Table 4.3). The retrospective examination indicated the parasite was present in the H&E sections but was not easily detectable after five minutes of searching. It is possible that the efficiency of detection for histological examination may be different for alternative personnel depending on their skill, level of experience and the length of time they spend examining the sections. The retrospective histological examination did not detect infection in six oysters described as infected by PCR and *in-situ* hybridisation. Overall, the PCR assay was found to be more sensitive than the initial histological examination for detecting the *Minchinia* sp. infecting rock oysters (Tables 4.3, 4.4 and 4.5). No statistically significant difference was detected between the PCR and ISH

assays and therefore PCR assay may provide an inexpensive method for detecting the parasite.

4.4.2 The 30 bp oligonucleotide ISH assay

The 30 bp oligonucleotide probe targeted a variable section of the SSU region of the parasite's rRNA gene in order to confer a higher level of specificity than the polynucleotide ISH probe. The oligonucleotide probe only recognized the *Minchinia* sp. infecting rock oysters. The probe did not recognize closely related haplosporidian parasites such as *Minchinia teredinis, Bonamia roughleyi, Haplosporidium costale or Haplosporidium nelsoni* or cross react with any of the host species tested (Figures 4.6, 4.7 and 8). Consequently, the ISH assay utilising the 30 bp oligonucleotide probe is likely to be species specific. It is impossible to ensure the assay does not react to uncharacterised or untested haplosporidian parasites. The assay was successful in localising individual parasites thereby detecting low levels of infection.

The comparison of diagnostic techniques summarised in Table 4.3 suggests the oligonucleotide ISH assay is more sensitive than the initial histological examination (Tables 4.3, 4.4 and 4.5). These data highlight the difficulty in detecting low numbers of relatively cryptic parasites in H&E sections that may be present in a variety of different tissue types. The oligonucleotide ISH assay detected the highest number of infected oysters in the comparison (29 from 56 oysters examined; Table 4.3). The sensitivity of the assay means the pathogen is detected earlier in the infection cycle and lower levels of infection can be detected. *In-situ* hybridisation costs considerably more that PCR and therefore it may be most suited to detecting the pathogen in low numbers of oysters where high sensitivity is required. Alternatively, in-situ

hybridisation could be used to confirm the results of high volume PCR screening, when histology proves difficult.

Further testing of rock oysters infected with *Minchinia* sp. is required to ensure the assay detects all strains of the parasite throughout its range. The oligonucleotide assay will be a useful confirmatory diagnostic test to ensure no contamination of PCR has occurred since it allows visualisation of the parasite within host tissues.

4.4.3 The polynucleotide ISH assay

A polynucleotide ISH probe was used by (Cochennec, N. *et al.*, 2000) to detect *Bonamia ostrea* in *Ostrea edulis* and was found to also detect *Bonamia* sp. in infected *Tiostrea chilensis* and *H. nelsoni* in infected *Crassostrea virginica*. The 144 bp polynucleotide probe obtained from the *Minchinia* sp. produced strong hybridisation signals with all of the haplosporidian parasites tested (*Minchinia* sp., *Minchinia teredinis*, *Haplosporidium nelsoni*, *Haplosporidium costale* and *Bonamia roughleyi*) and did not cross react with any host tissues in the five bivalve species tested (*Saccostrea cucullata*, *Saccostrea glomerata, Crassostrea virginica, Pinctada maxima and Teredo sp;* Figures 4.7 and 4.8*).* Further testing is required in order to assess whether the assay recognises basal or putative haplosporidian parasites such as the parasite detected in New Zealand paua, *Haliotis iris* (Diggles, B.K. *et al.*, 2002) or *Urosporidium* species. However, since the polynucleotide probe recognised three of the four Haplosporidia genera (*Minchinia*, *Haplosporidium* and *Bonamia*) the ISH assay may be a phylum specific test. The polynucleotide ISH probe failed to recognise *Marteilia* sp. from rock oysters (*Saccostrea cucullata*) or the Rhynchodid-like ciliate (Ciliophora). In addition, incidental parasites that did not belong to the

Haplosporidia, found in rock oysters (copepods, gregarines) did not cross-react (unpublished observations).

Disease has not been a major problem for the mollusc industries of Western Australia since major mortalities have not been reported for sometime. However, it is certain that many more pathogenic organisms remain to be discovered, especially as molluscs become subject to aquaculture or are subjected to environmental stresses associated with economic activity (Jones, J.B. and Creeper, J.H., 2006). Because of the age of the Australian continent and its relative isolation, many of these pathogens may prove to be unique to Western Australia (Jones, J.B. and Creeper, J.H., 2006). The polynucleotide ISH assay may be a useful tool for disease surveys of wild bivalve populations for haplosporidian parasites at low levels of infection. Many haplosporidians are known to cause considerable losses in naïve hosts following translocation (Burreson, E.M. and Ford, S.E., 2004; Burreson, E.M., Stokes, N.A. and Friedman, C.S., 2000b). Eradication of these parasites, once they have become established, is usually not feasible. The polynucleotide ISH assay is able to detect a variety of haplosporidian parasites in a variety of bi-valve species. Thus, it may provide target tissues for further analysis in the detection of undescribed or difficult to detect haplosporidian parasites at low levels of infection. Once a positive result has been obtained, the polynucleotide ISH assay must be used in conjunction with alternative assays (such as PCR, histological examination or electron microscopy) that will confirm the haplosporidian infection and allow speciation. It is possible that the polynucleotide assay may cross react with other untested parasite groups and consequently needs to be used with caution just as it is also possible for untested haplosporidian parasite species to cross react with the oligonucleotide probe.

Neither ISH assay recognised mature haplosporidian spores. The inability of the assay to detect mature spores is consistent with other haplosporidian parasites. Stokes et al., (1995a) and Stokes et al., (1995b) reported similar results in two haplosporidian species (*Haplosporidium nelsoni* and *Minchinia teredinis*). These findings were attributed to the inability of either the probe and/or the anti dioxigenin antibody to penetrate the spore wall. The lack of spore recognition may hamper the use of the assay in life cycle studies, such as the identification of an intermediate host. These results contrast with (Carnegie, R.B., Burreson, E.M., Hine, P.M., Stokes, N.A., Audemard, C., Bishop, M.J. and Peterson, C.H., 2006) whose ISH assay did recognise the spores of *Bonamia perspora*. Since *Bonamia perspora* is the only species of *Bonamia* recognized to produce spores it is likely that it's spores may enable penetration of the spore wall by either the probe and/or the anti dioxigenin antibody.

The PCR and *in situ* hybridisation tests developed and optimised in this study should prove a valuable research tool. Highly sensitive and reliable detection methods for the *Minchinia* sp. infecting rock oysters will facilitate and speed up further research into this parasite and the interactions between the parasite and its host.

Chapter 5 : Detection of *Minchinia* sp. in *Haplosporidium* sp. infected pearl oysters *Pinctada maxima* (Jameson, 1901).

This Chapter describes and discusses the results obtained when the probes assessed in Chapter 4 were applied to histologically positive samples of *Haplosporidium* sp. infected pearl oysters.

5.1 Introduction

The infection of pearl oysters, *Pinctada maxima*, attributed to a *Haplosporidium* sp. by Hine and Thorne (1998) has been detected on three occasions (Jones, J.B. and Creeper, J.H., 2006). The location of these infections was indicated in Figure 1.1. The prevalence of infection determined histologically in the Carnarvon outbreak was 4.0% (6/150) and in Cascade Bay in King Sound it was 4.7% (7/150). However, when the oysters from the latter outbreak were culled 15 days later the prevalence of infection had increased to 10.0% (Jones, J.B. and Creeper, J.H., 2006). The parasite is recognized as a significant concern for the pearling industry (Humphrey, J.D. and Norton, J.H., 2005; Humphrey, J.D., Norton, J.H., Jones, J.B., Barton, M.A., Connell, M.T., Shelley, C.C. and Creeper, J.H., 1998).

Chapter 3 demonstrated an infection by *Minchinia* sp. in rock oysters (*Saccostrea cucullata*) in pearl producing zones of Western Australia (Hine, P.M. and Thorne, T., 2002). The differential diagnosis of the rock and pearl oyster haplosporidians is based on the ultrastructural characteristics of the spores (Hine, P.M. and Thorne, T., 1998; Hine, P.M. and Thorne, T., 2002).

In an effort to determine whether *Haplosporidium* sp. infected pearl oyster spat from past outbreaks also contained *Minchinia* sp. previously detected in rock oysters (*Saccostrea cucullata*; Chapter 3) a polymerase chain reaction (PCR) and *in situ* hybridisation (ISH) assay for *Minchinia* sp. was used to analyze haplosporidians in archived tissues from *Haplosporidium* sp. infected pearl oysters.

5.2 Methods and materials

Representative histologically-positive paraffin-embedded pearl oyster spat infected with *Haplosporidium* sp. Hine and Thorne, 1998 were obtained from archived cases of previously reported outbreaks (Hine, P.M., 1996; Hine, P.M. and Thorne, T., 1998; Jones, J.B. and Creeper, J.H., 2006). The original geographic location of the source material is shown in Figure 1.2. Formalin fixed paraffin embedded samples of *Minchinia* sp. in *S. cuccullata* were obtained from the Montebello Islands as described in Chapter 2.

In order to assess whether *Minchinia* sp. was present in *Haplosporidium* sp. infected pearl oysters, archived formalin fixed samples of pearl oysters infected with the parasite were subjected to PCR. Overall, twelve histologically positive *Haplosporidium* sp. samples were analysed. Six of these samples were from Carnarvon, four were from Cascade Bay and two were from Willie Creek. Two regions were identified for amplification. The Minch F1B/R2B primers encompass a variable section of the small ribosomal subunit (SSU) region of the parasite's rRNA gene while an additional amplification was performed using the SSF66 and SSR69 primers.

5.2.1 DNA extraction: Archived samples.

Genomic DNA was extracted from the formalin-fixed paraffin embedded pearl oysters using the freeze thaw method outlined in Chapter 3. The quality of the genomic DNA and absence of inhibitory factors from the extractions was verified using PCR with bivalve primers 16R3 5'-GCT GTT ATC CCT RNR GTA-3' and Proto 16'F'-AWK WGA CRA GAA GAC-3' (Chase, M.R. *et al.*, 1998).

5.2.2 Amplification by Polymerase Chain Reaction.

Histologically positive *Haplosporidium* sp. samples from the Willie Creek and Carnarvon infection sites were amplified using Minch F1B and Minch R2B primers. These primers amplify a 144 bp section within the SSU region of the rRNA gene from *Minchinia* sp. infecting *Saccostrea cucullata* on the same coastline as the pearl oyster parasite. The Minch F1B and R2B primers encompass a variable section within the SSU region of the rRNA gene.

Each PCR was performed using the method outlined in Chapter 3. An additional measure was taken to ensure the absence of contamination from genomic *Minchinia* sp. DNA in the F1B/R2B reactions. This measure involved the attempted amplification of a 754 bp section of the Minchinia sp rRNA gene using the FSSUF/SSR69 primers. A positive amplification from these primers would indicate contamination since formalin fixation results in the cross linking of histones and fragmentation of sample DNA making amplification of sequences larger than 300 to 500 bp difficult (Paabo, S., 1990). Thos primer combination was assessed using genomic rock oyster DNA to ensure successful amplification of the target sequence. The reaction conditions for the FSSUF/SSR69 reactions were as follows: Reaction mixtures had a total volume of 25 µL and contained reaction buffer (67 mM Tris-HCl, 16.6 mM $[NH_4]_2SO_4$, 0.45% Triton X-100, 0.2 mg/mL Gelatin and 0.2 mM dNTP's), 2 mM of $MgCl_2$, 40 pmol of each primer, and 0.55 units of *Taq* polymerase and template DNA. Each of the reaction mixtures was subjected to (i) an initial denaturation phase of 5 min at 94°C, (ii) 35 amplification cycles, each cycle consisting of 30 sec of denaturation at 94°C, 30 sec of annealing at 59°C, 4 minutes of extension at 68°C and (iii) a final 7 min

extension at 68°C. Polymerase chain reaction products were visualised by loading a 10 µL aliquot of the products on a 2% agarose gel and electrophoresing it for 20 min at 90 volts. Detection of the PCR products was performed using ethidium bromide staining.

Given the assessment of potential contamination by the FSSUF/SSR69 reactions may be confounded if the source of contamination were shorter gene fragments from the F1B/R2B PCR and cloning reactions an alternative region was selected for amplification. This assay was required since the F1B/R2B reactions were commonly used in this laboratory to assess rock oyster samples for *Minchinia* sp. Consequently, potential contamination by shorter rRNA gene fragments, such as those from the F1B/R2B PCR reactions were assessed by using the primers designated SSF66 and SSR69 (Chapter 4). These primers were designed to target a 220 bp sequence (Appendix 1) so that amplification from formalin fixed tissues could be reliably performed. The successful amplification of a 220 bp region ensured the absence of contamination from shorter PCR products in the Minch PCR reactions. The SSF66/SSR69 primers target variable regions of the parasite's SSU rRNA gene but encompass a conserved region. Amplification conditions were the same as for the Minch primers except the annealing temperature was raised to 62 °C. The specificity of the SSF66/SSR69 primers was assessed with *H. nelsoni* and *H. costale* DNA.

Table 5.1 Primer sequences employed in Chapter 5.

Primer	Sequence (5' -3')	Position*	Reference
Minch F1B	ctc gcg ggc tca gct t	1295	Chapter 3
Minch R2B	ggc gct ttg cag att ccc ca	1439	Chapter 3
SSF66	ccg ccg atg ccc agc cgt at	551	Chapter 4
SSR69	agc cca aaa cca aca aaa cgt cca ca	754	Chapter 3
FSSUF	ctc aaa gat taa gcc atg cat gtc caa gta ta	#	Chapter 3
SSRDb#	gtt agc ctt gcg cgc agc cga tac g	567	This Chapter

*Position on the entire *Minchinia* sp. *SSU rRNA gene sequence Genbank accession: EF 165631.* SSRDb was used as an ISH probe only. SSR69 was used as both a PCR primer and an ISH probe. # FSSUF primer targets a region before EF165631.

Each PCR was performed using the method outlined in Chapter 3. In order to ensure no contamination with genomic *Minchinia* DNA had occurred primers which target a 754 bp section of the *Minchinia* sp. rRNA gene were employed. These reactions were performed in addition to the usual negative controls employed in all PCR reactions. These primers (FSSUF and SSR69; Table 5.1) were used to amplify a section of the *Minchinia* sp. in Chapter 3. A positive amplification from these primers would indicate contamination since formalin fixation results in the cross linking of histones and fragmentation of sample DNA making amplification of sequences larger than 300 to 500 bp difficult (Paabo, S., 1990). Potential contamination by shorter rRNA gene fragments, such as those from PCR reactions were assessed by using the primers designated SSF66 and SSR69 (Table 5.1). The SSF66/SSR69 primers target variable regions of the parasite's SSU rRNA gene but encompass a conserved region. These primers were assessed in Chapter 4 and were found not to react to closely related haplosporidian species *H. nelsoni* and *H. costale*. The SSF66/SSR69 primers target a 220 bp sequence so that amplification from formalin fixed tissues could be reliably performed. The successful amplification of a 220 bp region ensured the absence of

contamination from shorter PCR products in the Minch F1B/R2B PCR reactions. Amplification conditions were the same as for the Minch primers except the annealing temperature was raised to 62 ^{o}C.

Polymerase chain reaction was also used to attempt to amplify *Haplosporidium* sp. DNA from the histologically positive pearl oyster samples. These reactions were performed using all combinations of the HAP primers (Renault, T., Stokes, N.A., Chollet, B., Cochennec, N., Berthe, F., Gerard, A. and Burreson, E.M., 2000) and at least 10 other primer combinations of novel design utilising 16 s, and protozoan (Carnegie, R.B., Meyer, G.R., Blackbourn, J., Cochennec-Laureau, N., Berthe, F. and Bower, S.M., 2003) primers with proximate target regions suitable for amplification from formalin fixed material (Appendix 2).

5.2.3 DNA sequencing

DNA cloning and sequencing was performed using the method outlined in Chapter 3. Three cloned DNA inserts were each sequenced for each positive PCR. Primer sequences were removed from each end of the resulting consensus sequence and the identity, position and direction of the PCR products were verified by directly comparing to the *Minchinia* sp. SSU rRNA sequence from *S. cuccullata*. Additional comparisons were also made using BLAST searches of GenBank database: (http://www.ncbi.nlm.nih.gov/blast/).

5.2.4 In-situ hybridisation

In addition to the precautions taken to ensure the absence of contamination in the PCR reactions, ISH was also performed. *In situ* hybridisation was performed on formalin-fixed, paraffin-embedded tissue collected at the Carnarvon, Cascade Bay and

Willie Creek infection sites. *In situ* hybridization was performed using the 30 bp oligonucleotide probe (SSR69) developed in Chapter 3 and assessed in Chapter 4. Positive controls included sections of rock oyster infected with *Minchinia* sp. collected from the Montebello Islands in July 2005. Negative controls used sections containing no DNA in the hybridisation mix as well as uninfected pearl oyster spat obtained from the Broome hatchery. The *in situ* hybridisation procedure was performed using the method outlined in Chapter 3. The specificity of the probe was monitored using *Haplosporidium costale* and *Minchinia teredinis* sections included in all of the assays (Figure 5.6). These parasites were chosen for specificity tests since they possess the closest known sequences to the SSR69 probe. In order to confirm the results of the SSR69 probe another oligonucleotide probe was designed. The new probe was designated SSRDb and its sequence was 5' GTT AGC CTT GCG CGC AGC CGA TAC G 3'. The SSRDb probe targets a highly variable region on the *Minchinia*'s SSU rDNA sequence (positions 567-588; Appendix 1). The new probe was commercially synthesized and labelled with dioxigenin at the 3' end (Operon Technologies, Germany). Specificity of the SSRDb probe was assessed and monitored using *Haplosporidium costale*, *Haplosporidium nelsoni* and *Minchinia teredinis* sections. The procedure used for the SSRDb ISH assay was identical to that used for the SSR69 ISH assay and is described in Chapter 4. However, the procedure was varied for the pearl oyster samples since many of the heavily infected the digestive glands of these samples would disintegrate during the ISH procedure resulting in the loss of a considerable number of samples. This problem was overcome by reducing the Proteinase K digestion time to 15 minutes.

5.2.5 Genealogical comparisons of the SSU rRNA sequences

The phylogenetic relationships between SSU rRNA sequences of *Minchinia* sp. were compared to other *Minchinia* spp. using distance based trees and an multidimensional scaling (MDS) analysis of pairwise comparisons of genetic distances performed using the software SPSS 13.0.

5.2.5.1 Interspecific relationships.

The SSU region of the rRNA gene sequences used in the distance comparison were obtained from Genbank. The sequences used belonged to *Haplosporidium nelsoni* (U19538), *Minchinia teredines* (U20319), *Minchinia tapetis* (AY449710), *Minchina chitonis* (AY449711), *Minchinia* sp. from *Cyrenoida floridana* (AY449712), *Bonamia ostreae* (AF262995), *Bonamia exitiosa* (AF337563). Sequences were aligned using CLUSTALW (Thompson, J.D., Higgins, D.G. and Gibson, T.J., 1994) in MEGA 3.1 software (Kumar, S., Tamura, K. and Nei, M., 2004).

Distance based trees were constructed using the MEGA 3.1 computer program (Kumar, S., Tamura, K. and Nei, M., 2004) with a neighbour-joining algorithm (Saitou, N. and Nei, M., 1987). The tree was constructed in order to highlight the relationships between the sequences obtained in this study and those of closely related parasites in the *Bonamia* and *Minchinia* genera. The neighbour-joining method of tree construction is well suited to the situation where one does not know whether rates of substitution are constant across all clades of the tree as was the case in this study (Saitou, N. and Nei, M., 1987). An alternative method of tree construction is the maximum parsimony approach. However, the lack of a meaningful outgroup in this chapter of the study invalidates this procedure. Nodal support was determined by 1000 replicate bootstrap analyses.

The bootstrap analysis method to assess confidence in a tree.

A bootstrap test was used to assess confidence in the nodes of the neighbour joining tree. This method uses a subset of the original data and, from this new data set, a new tree is constructed (Felsenstein, J., 1985). Each node in the original tree is compared to the new tree to determine whether the tree has the same cluster of sequences. This whole procedure of resampling the data, drawing a tree, and tallying up nodes that are in the original tree is repeated perhaps a 1000 times (Felsenstein, J., 1985). The final result is shown graphically as a number next to each node which indicates the percentage of times that the cluster is present among the resampled trees. If that percentage is high, then it is more likely that the cluster actually does belong together.

5.2.5.2 Intraspecific variation: The Minimum Spanning Network.

Relationships amongst the SSU rRNA sequences were explored using a minimum spanning network (MSN). An MSN, describing the nucleotide substitutions amongst the SSU rRNA alleles was constructed by statistical parsimony, as applied in the software TCS 1.18 (Clement, M. *et al.*, 2000). A network can more clearly and precisely reproduce the evolutionary relationships of closely related, intra-specific alleles, particularly as ancestral alleles can continue into existing populations (Templeton, A.R., 2001). Networks can also include both indels and missing alleles such as extinct or unsampled alleles in the substitution pathways between alleles. In addition, networks can include information about the existence of alleles through evolutionary time that can be inferred from inter-alleles relationships (Templeton, A.R., 1998) and can be used to compare genetic and geographic distances.

Once the necessary input file was constructed for the TCS 1.18 software, a parsimony connection limit of $P = 0.05$ was used in the construction of the network. The output was untangled so that no SSU rRNA alleles crossed base substitutions. Nucleotide substitutions were displayed on the network along with the base change, type and location. The network was then applied to a map indicating the location from which the sequences were obtained.

5.3 Results

5.3.1 Polymerase Chain Reaction

The reactions with the Minch F1B/R2B primers produced consistently positive results with no indication of contamination (Figures 5.1 and 5.2). Of the twelve histologically positive pearl oysters samples tested, five were positive by PCR (Table 5.2). Of the six histologically positive samples from Carnarvon three produced positive PCR results while both of the Willie Creek samples produced positive PCRs (Table 5.2). No *Minchinia* sp. sequences could be obtained from the four histologically positive Cascade Bay samples. While these samples were negative by ISH, samples from Cascade Bay did produce positive ISH results with the SSRDb and SSR69 probes at low levels of intensity (SSRDb 8/30; 26.7%; SSR69 30/94 36%). Overall, three clones were obtained from each of the positive PCR samples to produce fifteen sequences.

Table 5.2 Summary of number of samples tested and a comparison of results from PCR and ISH assays.

Location	n	Positive PCR	ISH-SSR69	ISH-SSRDb
Cascade Bay	4	0	4	0
Cascade Bay 2	30*	N/A	N/A	8
Carnarvon	6	3	6	3
Willie Creek	2	2	2	2

* Second batch of archived Cascade Bay oysters were histologically negative although retrospective examination of the ISH (SSRDb) positive sections revealed haplosporidian parasites in the affected tissues.

Attempts to detect contamination with genomic *Minchinia* sp. DNA by amplifying a 754 bp section of the *Minchinia* sp. rRNA gene using the FSSUF and SSR69 primers failed (Figure 5.2). This result is consistent with observations that amplification of formalin fixed tissues above 200-500 bp is difficult. In addition, the SSF66/SSR69 primers that target a different 220 bp region were successful in obtaining a positive reaction from

the Willie Creek and Carnarvon samples ensuring the absence of contamination from shorter PCR products in the Minch PCR reactions.

When the 144 bp sequences were compared to the sequences of *Minchinia* sp. from *S. cuccullata*, the genetic distances between the sequences were considerably less than the distance between known species for this region (Figures 5.3 and 5.4). The region targeted by the Minch primers is a variable section of the parasite's SSU rRNA gene. Four sites within the 144 bp SSU rRNA sequence produced by the pearl and the archived rock oyster parasites described by Hine and Thorne (2002) were different to that produced by the *Minchinia* sp. (GenBank accession number: EF 165631). Base 1389 on EF165631 were different in all parasite SSU rRNA sequences from Carnarvon which had a T at this site rather than the C present in the other samples (Appendix 1). Both the Carnarvon and the sequence obtained from the rock oyster samples described by Hine and Thorne (2002) had a G substituted for an A at base 1359 on EF 165631. One of the Carnarvon sequences had a T at base 1311 rather than the C present in all other samples. Both Willie Creek samples were identical to the 2005 rock oyster samples except base 1315 had an A substituted for a G.

The sequences produced by the SSF66/SSR69 primers from the Willie Creek and Carnarvon samples were identical to that obtained from the *Minchinia* sp. infecting rock oysters from the Montebello Islands. Consequently, the sequences obtained from the Willie Creek samples had a known sequence of 364 bp with only a single base pair difference with the *Minchinia* parasite.

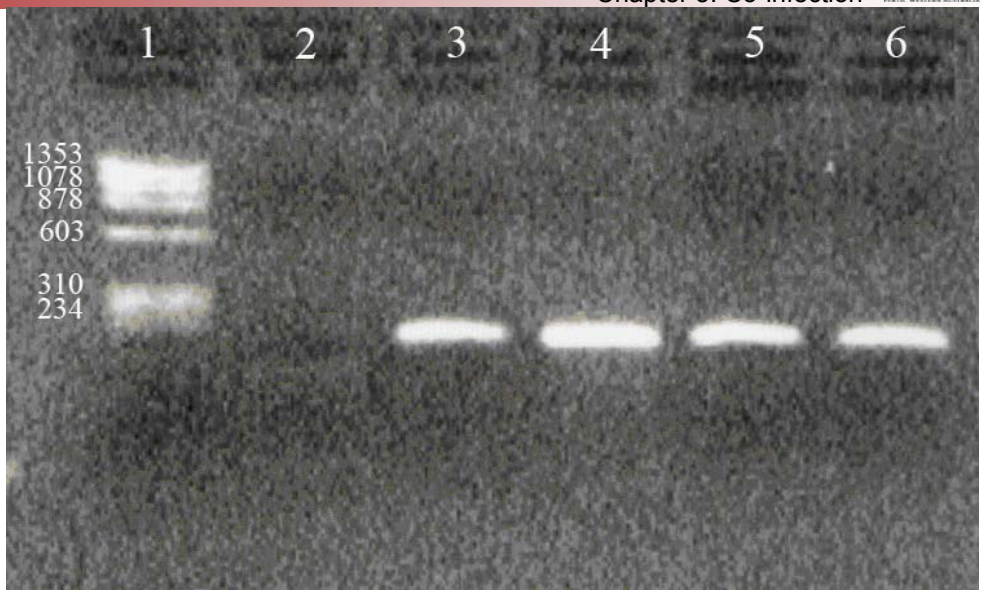


Figure 5.1 Agarose gel electrophoresis of formalin pearl oyster tissue from each of the infection sites. Samples were electrophoresed in a 2% agarose gel for 20 minutes at 90V. Lane 1 contains *Hin*dIII/Phi markers, size of markers are indicated on the left. Lane 2 is the negative control (no DNA), Lane 3 contains haplosporidian DNA from the Carnarvon site, Lane 4 contains haplosporidian DNA also from the Carnarvon site, Lane 5 contains haplosporidian DNA from the Willie creek site. Lane 6 contains haplosporidian DNA also from the Willie Creek site. DNA was stained with ethidium bromide and visualised using UV light.

Attempts to obtain *Haplosporidium* sp. DNA from the histologically positive samples utilising the HAP (Renault, T., Stokes, N.A., Chollet, B., Cochennec, N., Berthe, F., Gerard, A. and Burreson, E.M., 2000), 16 s (Medlin, L., Elwood, H.J. and Sogin, M.L., 1988), Protozoan (Carnegie, R.B., Meyer, G.R., Blackbourn, J., Cochennec-Laureau, N., Berthe, F. and Bower, S.M., 2003) and novel primers (Appendix 2) were unsuccessful in producing any *Haplosporidium* sp. sequences.

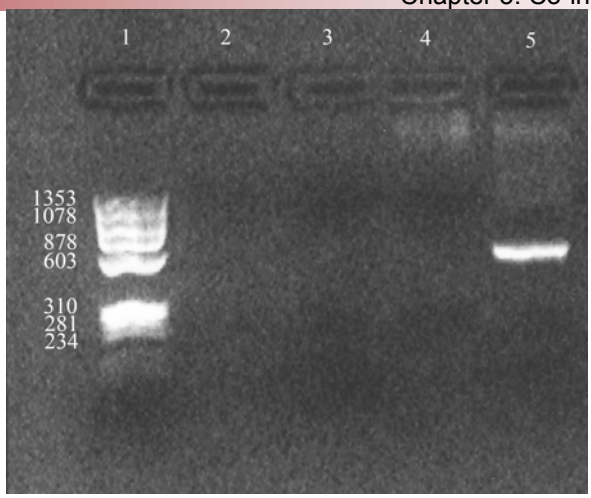


Figure 5.2 Agarose gel electrophoresis of the FSSUF and SSR69 primers and formalin fixed pearl oyster tissue from the Willie Creek infection site. Samples were electrophoresed in a 2% agarose gel for 20 minutes at 90V. Lane 1 contains *Hin*dIII/Phi markers, size of markers are indicated on the left. Lane 2 is the negative control (no DNA). Lanes 3 and 4 contains haplosporidian infected pearl oyster DNA from the Willie Creek site. Lane 5 contains *Minchinia* sp. infected genomic oyster DNA from the Montebello Islands (positive control). DNA was stained with ethidium bromide and visualised using UV light.

Interspecific comparison with other Minchinia species.

Overall, the genetic distance between the pearl oyster parasite PCR products was of the same magnitude as the genetic distance between the pearl oyster PCR products compared to the rock oyster PCR products (Figures 5.3 and 5.4). No sequences could be obtained from the Cascade Bay samples.

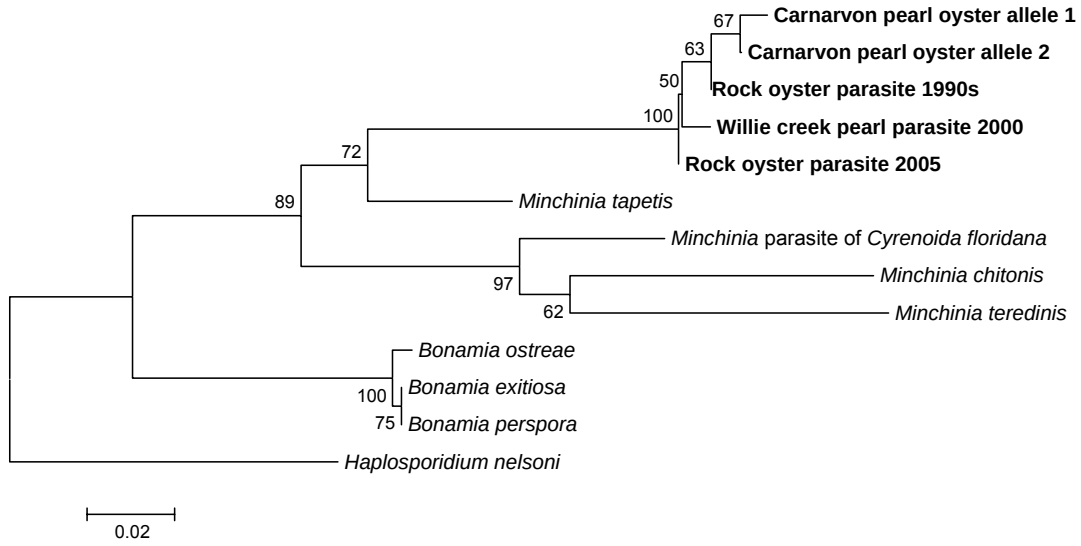


Figure 5.3 Neighbour Joining tree illustrating the relationships among the 144 bp SSU rRNA gene sequences from phylum Haplosporidia. Tree was constructed with a Kimura 2-parameter algorithm. The scale bar represents the equivalent of 0.02% nucleotide sequence divergence. Nodes are supported by a 1000 replicate bootstrap analysis.

In order to compare the spatial patterns of the SSU rRNA gene sequence differentiation among the samples, the multi-dimensional scaling (MDS) method (Hair, J.F. *et al.*, 1992) was used to map the genetic distance between each pair of samples in two-dimensional space.

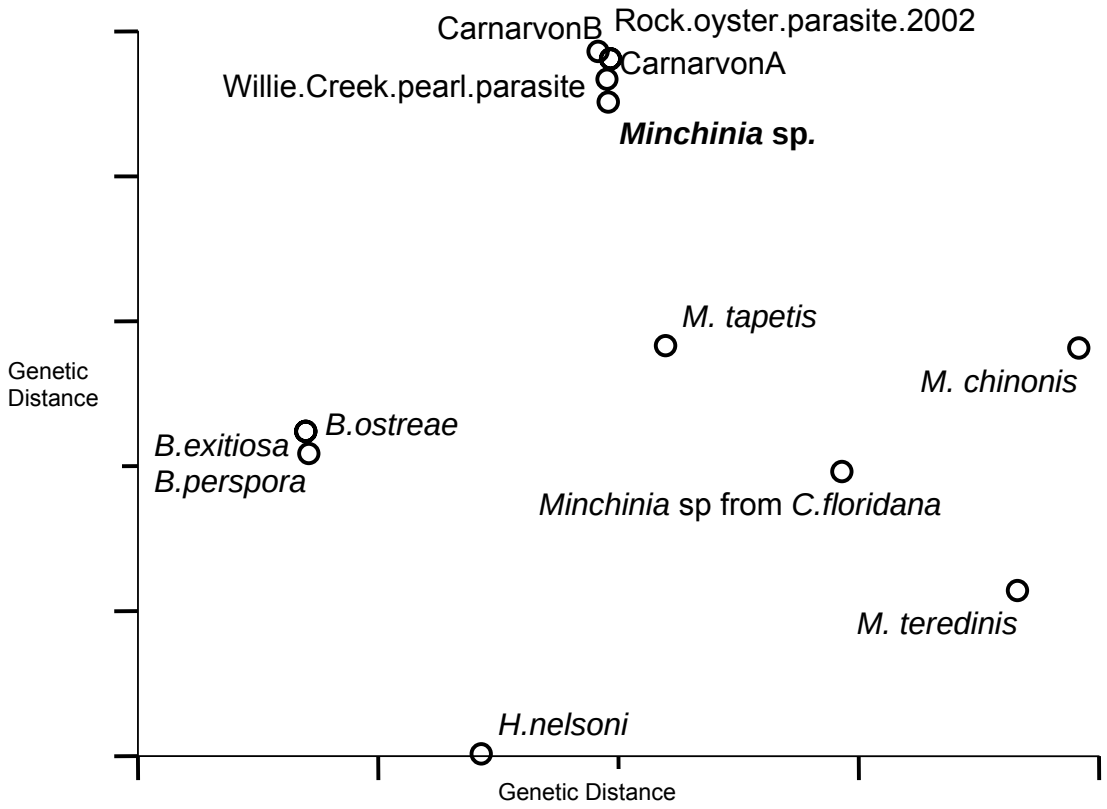


Figure 5.4 A two-dimensional ordination of the pairwise genetic distances obtained from the rock and pearl oyster samples. Carnarvon represents the haplosporidian sequence obtained from Carnarvon pearl oyster spat. Rock oyster parasite 2002 represents the sequence obtained from the samples described by Hine and Thorne (2002). Willie Creek represents the sequence obtained from the Willie Creek haplosporidian sample. *Minchinia* sp. represents the original sequence obtained in Chapter 3. Stress level = 0.08.

The MDS clearly shows the pearl oyster sequences grouping closely together with the rock oyster samples and are distinct from the rest of the *Minchinia* and are even more distant from the *Bonamia* and the *Haplosporidium* (Figure 5.4).

5.3.2 In situ Hybridisation

In situ hybridisation was performed in order to validate the sequences obtained by PCR from the infected pearl oyster tissues and confirm the positive PCRs were not the result of contamination. Parasite identity and location in tissue sections was confirmed

in hematoxylin-eosin sections cut adjacent to those tested with *in situ* hybridisation (Figures 5.5 and 5.6). The SSR69 *in situ* hybridisation assay yielded positive reactions from all of the parasite-infected pearl and rock oysters (Figures 5.5 and 5.6). As in Chapter 4 the assay did not hybridise to *Haplosporidium costale, H. nelsoni* or *Minchinia teredinis* infected oyster tissues.

Since it is impossible to rule out a chance cross reaction with an undescribed haplosporidian species such as the pearl oyster parasite, a second oligonucleotide ISH probe was designed. The new probe was designated SSRDb. The SSRDb ISH assay yielded positive reactions from the haplosporidian infected rock oysters but only some of the haplosporidian parasites in infected pearl oysters produced a positive reaction (Figure 5.7). The SSRDb probe did not react to closely related haplosporidian species *Haplosporidium costale*, *Haplosporidium nelsoni* or *Minchinia teredinis* (Figure 5.8).

The cells reacting to the SSRDb probe were detected in the connective (Leydig) tissue surrounding the digestive gland and less commonly in the mantle and gills of infected oysters. The cells consisted predominately of single; bi- and tri- nucleate life stages and were approximately 7 µm in diameter. No phagocytosis of parasite cells or necrotic debris was observed despite the presence of granulocytes among the parasites. The intensity of infection of the SSRDb positive cells was considerably lower when compared to the cells detected by the SSR69 probe (Figure 5.6 and 5.7).

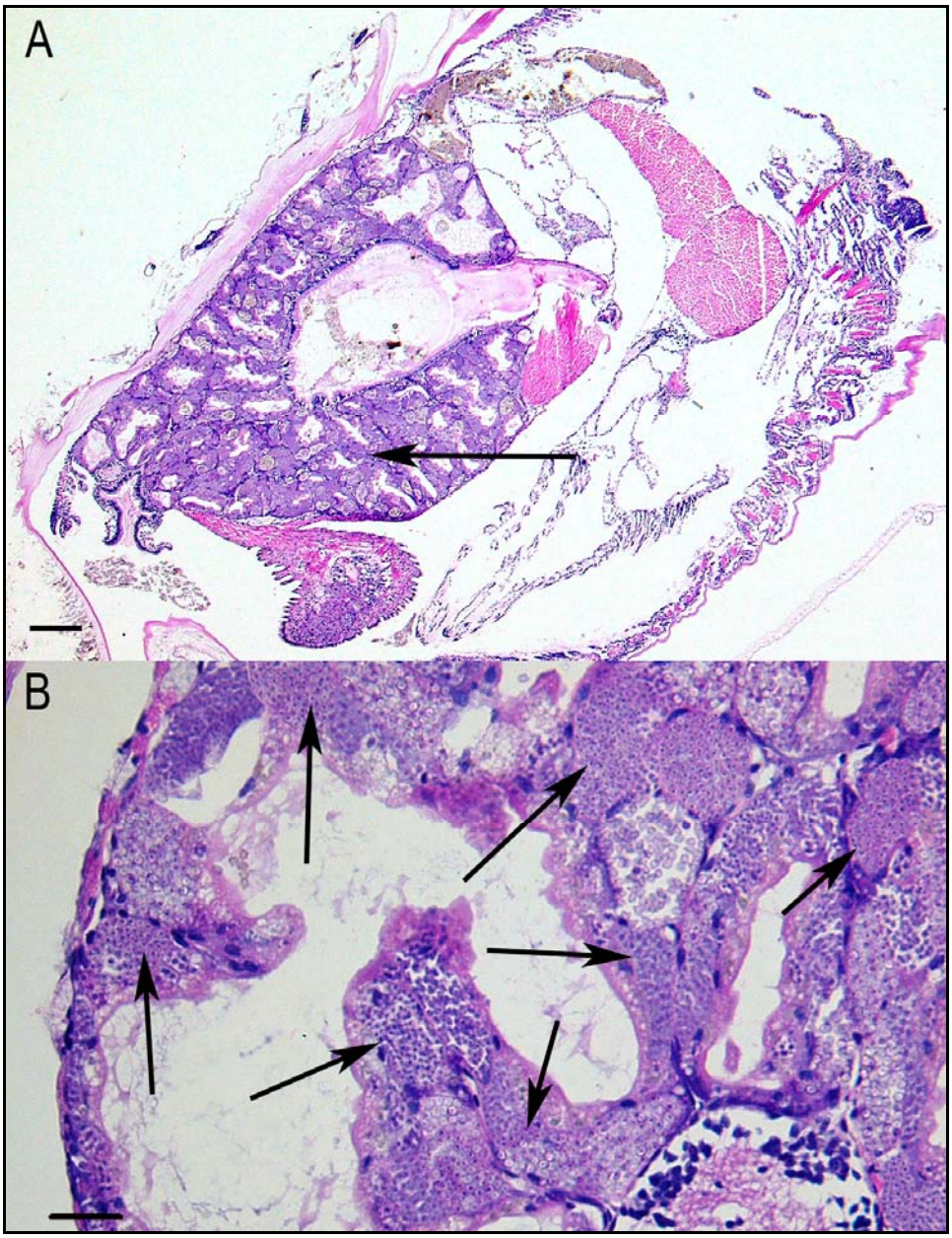


Figure 5.5 Pearl oyster tissue containing haplosporidian parasites in a hematoxylin-eosin stained section. (A): Entire oyster with a heavily infected digestive gland (arrow). Scale bar = 100 µm. (B): Higher magnification view of the same pearl oyster. Several life stages of the haplosporidian parasite are present. Scale bar = 30 µm.

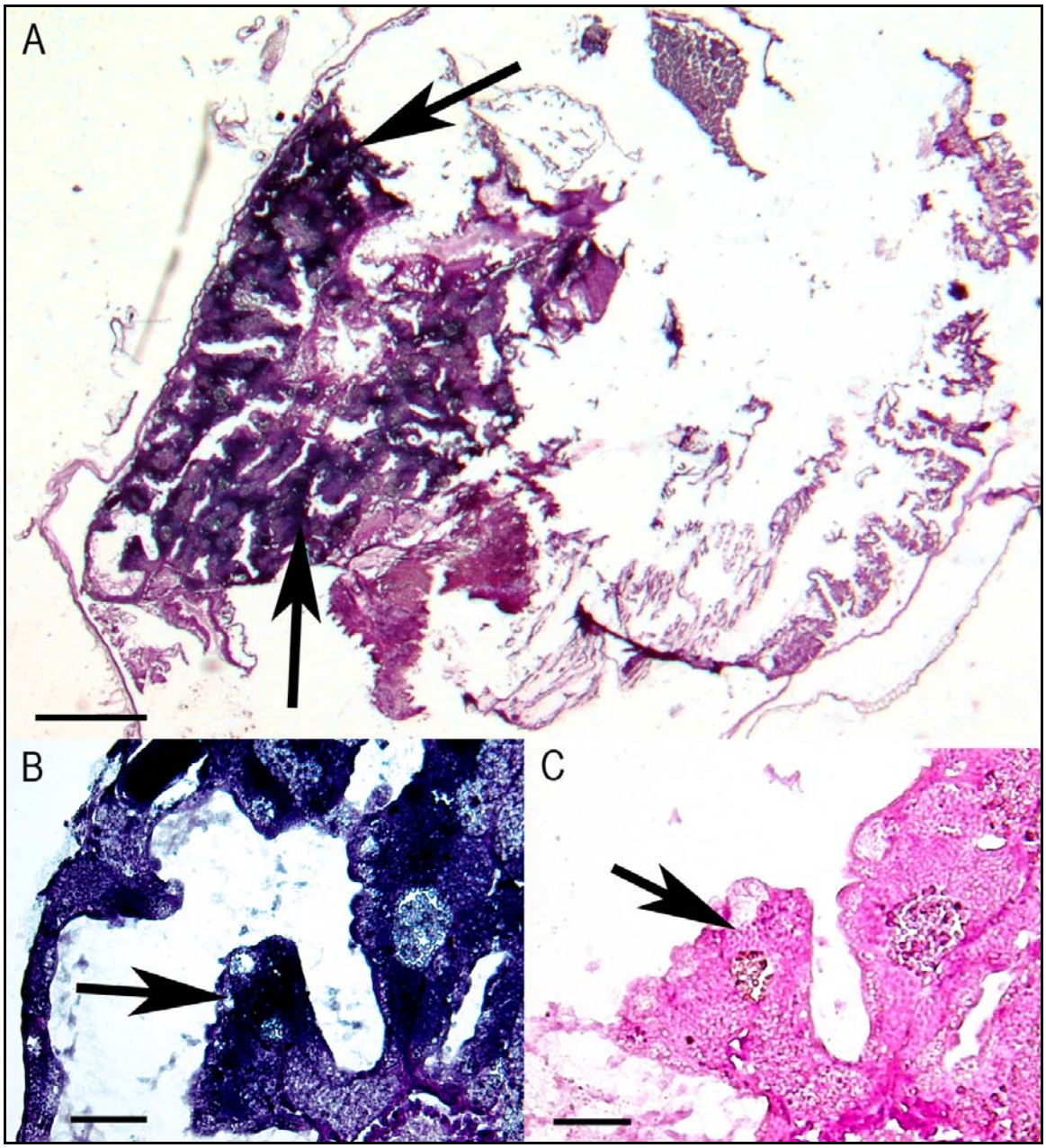


Figure 5.6 Haplosporidian parasites (arrows) identified in an *in situ* hybridisation of pearl oyster digestive gland. (A): *In situ* hybridisation containing the SSR69 probe. Parasites are identified by a darker colouration. Scale bar = 0.5 mm. (B): higher magnification view of the same section. Scale bar = 30 µm. (C): Negative control (no probe) serial section from the same *in situ* hybridisation. Sections are counterstained in a brazilin hematoxylin. Scale bar = 30 µm.

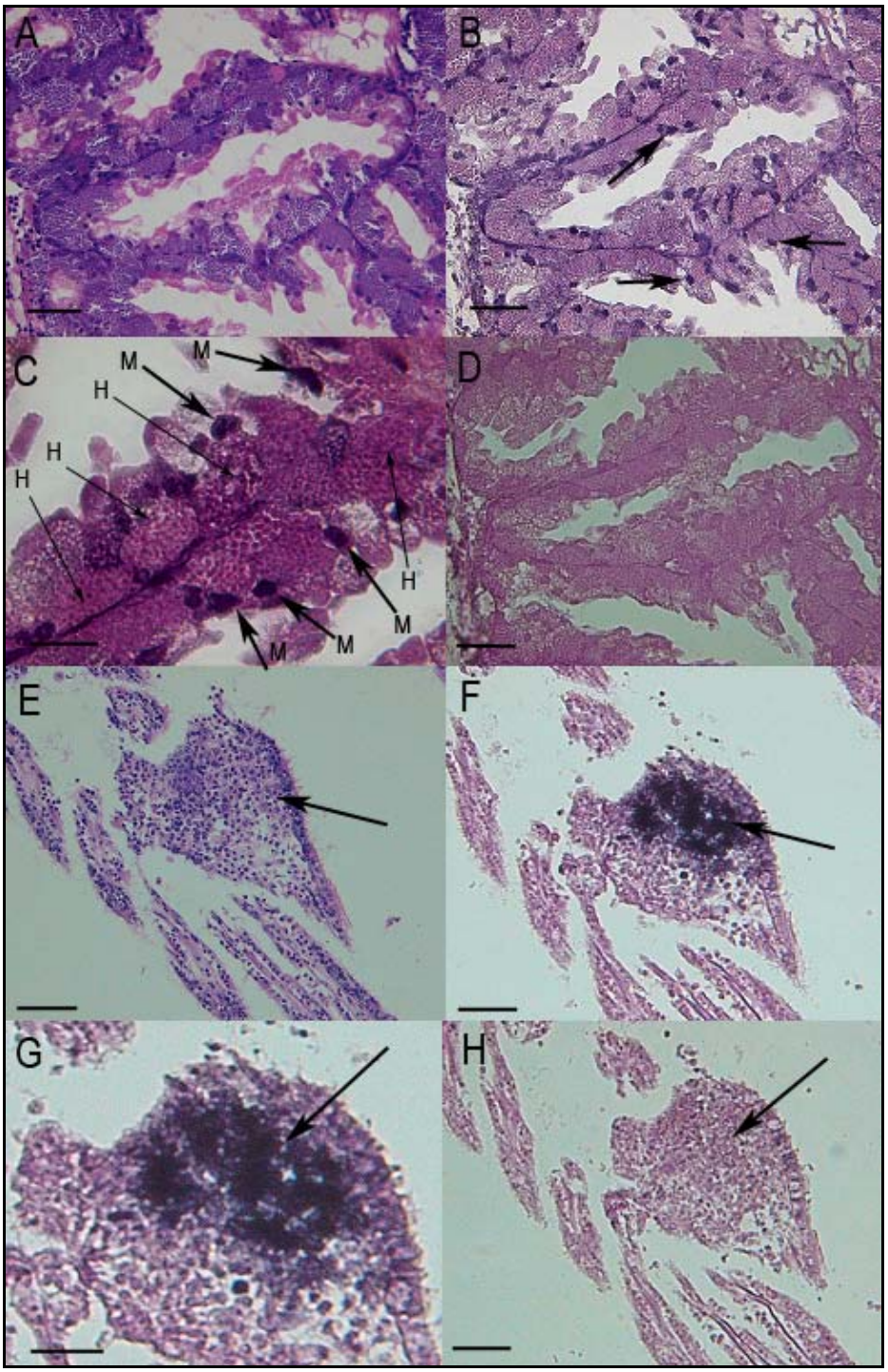


Figure 5.7 Haplosporidian parasites (arrows) identified in an *in situ* hybridisation of pearl oyster digestive gland. (A): H/E stained section of an infected pearl oyster digestive gland. (B) Serial *in situ* hybridisation containing the SSRDb probe. Scale bar = 65 µm (C): Higher magnification view of the same section. Arrow with an M denotes a *Minchinia*, Arrow with an H denotes *Haplosporidium hinei*. (D): Negative control serial section containing an irrelevant (MSX) probe. Scale bar = 65 µm. (E): H/E stained section of an infected rock oyster gill section. Scale bar = 40 µm. (F) Serial *in situ* hybridisation containing the SSRDb probe. Scale bar = 40 µm (G): Higher magnification view of the same section. (H): Negative control serial section containing an irrelevant (MSX) probe. Scale bar = 40 µm. Sections are counterstained in a brazilin hematoxylin.

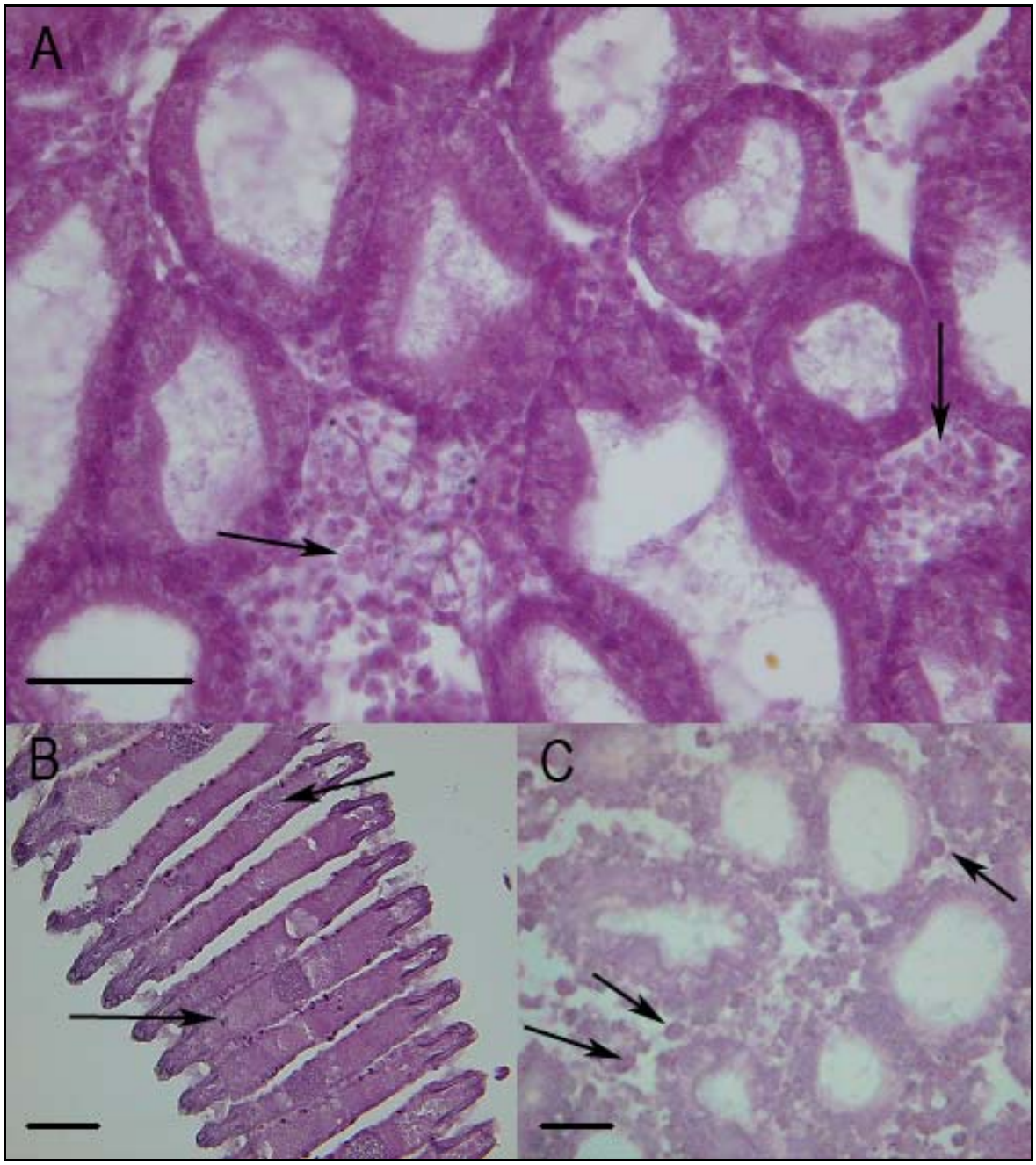


Figure 5.8 The bi-valve sections used to assess the specificity of the SSRDb ISH assay. Arrows indicate example parasites. (A) *Haplosporidium nelsoni* infected Eastern oyster (*Crassostrea virginica*) digestive gland. Scale bar = 35 µm, (B*) Minchinia teredinis* in *Teredo* sp. gill. Scale bar = 40 µm. (C) *Haplosporidium costale* infected Eastern oyster digestive gland. Scale Bar = 20 µm.

Intraspecific variation among the Minchinia sp. *alleles*

Enough polymorphic sites were found within the Minch SSU rRNA sequences to allow some preliminary assessment of intraspecific genetic variation. Overall, a 144 bp portion of the SSU rRNA gene was sequenced for 17 individuals from five sampling locations (including archived rock oyster samples). The five polymorphic sites were identified in the SSU rRNA sequences which led to the identification of five different alleles among the 17 individuals sampled from various hosts. The evolutionary relationships among the haplosporidian SSU rRNA alleles were estimated by constructing a network using the parsimony method of Templeton (1998). The network was imposed onto a map of the region where the infections and the resulting sequences were detected and is displayed in Figure 5.9.

Slightly different groups were recognisable in the MSN when compared to both the neighbour-joining tree (Figure 5.3) and MDS analysis (Figure 5.9). The network more clearly illustrates the nature of the relationships among the alleles (Figure 5.9). Unlike the distance based tree, the MSN also showed a tendency for the SSU rRNA sequences from the same geographic region to cluster together rather than sequences from each host to group together. The main findings of the MSN were:

- Sequences grouped on the basis of geographic region rather than by host species.
- Sequences were also grouped according to the year of sampling.
- The rock oyster parasite SSU rRNA sequences were between the pearl oyster SSU rDNA sequences from the two different regions.

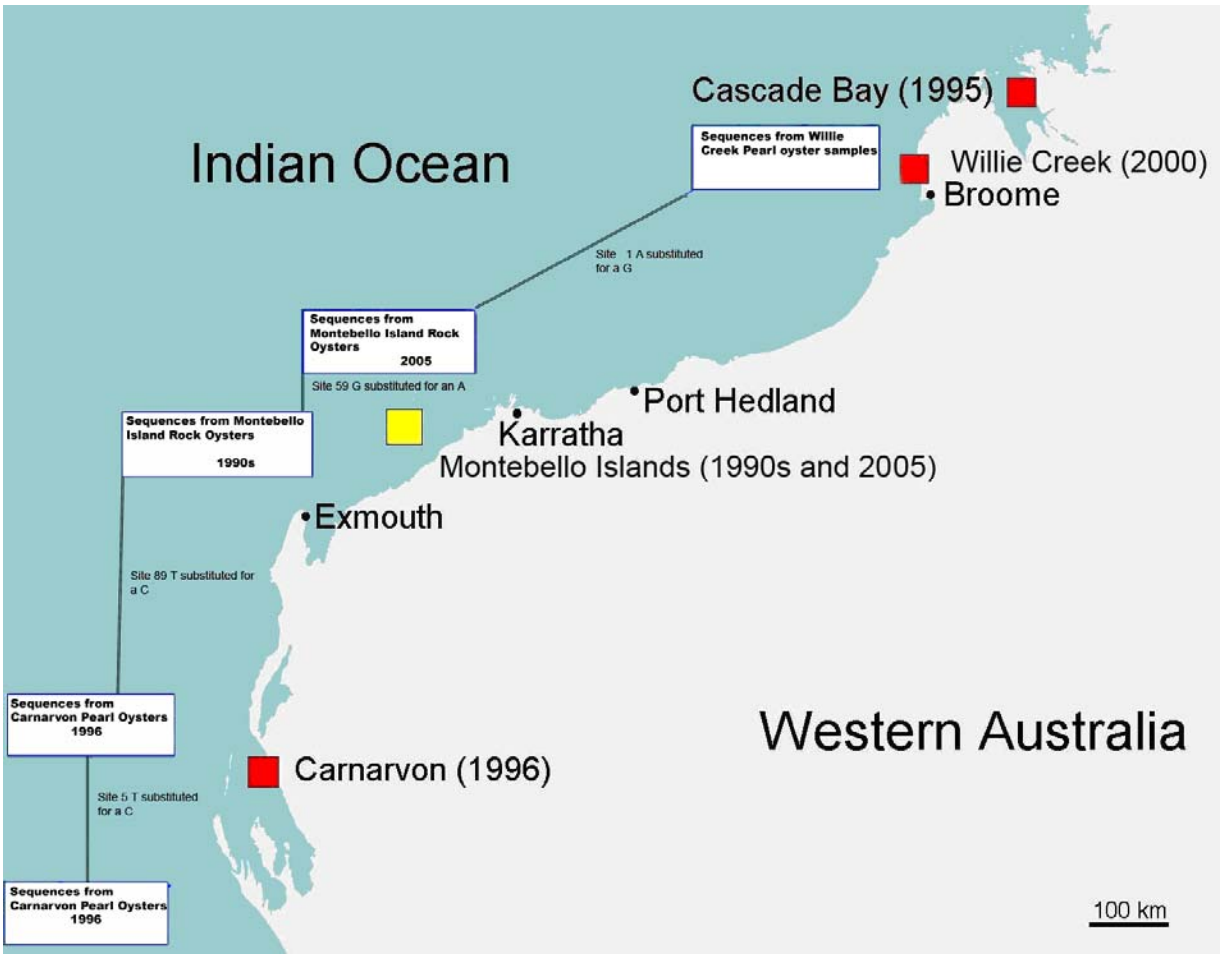


Figure 5.9 Minimum spanning network of the SSU rRNA gene sequences obtained from the rock and pearl oyster parasites. The white rectangles represent extant parasite SSU rRNA alleles. Site indicates position on the Minch F1B/R2B sequence (Appendix 1).

5.4 Discussion

The results of this study indicate that some *Haplosporidium* sp. infected pearl oysters were also infected with a *Minchinia* sp. recently identified in rock oysters from the same coastline (Hine, P.M. and Thorne, T., 2002). The results of DNA-based diagnostic assays supported each other. The 144 bp PCR products obtained from the Willie Creek samples encompass a variable region of the *Minchinia* SSU rRNA gene yet it differs from the rock oyster parasite by only one base pair. The results of the same assay from the Carnarvon sample differed by only two base pairs. Positive PCRs were obtained using the SSF66/SSR69 primers which target two other variable SSU rDNA regions. The SSF66/SSR69 PCR assay produced negative results when tested with *H. nelsoni* and *H. costale* infected oyster DNA. When the sequence obtained from the SSF66/SSR69 regions is added to the 144 bp sequence obtained from the F1B/R2B reactions, the Willie Creek sample has a total of 364 bp of known sequence with only a single base pair different from the rock oyster parasite. The PCR produced consistent results with no indication of contamination. Attempts to target a larger region with the primers FSSUF and SSR69 failed. This is consistent with observations that formalin fixation makes amplification of regions larger than 200-500 bp difficult due to fragmentation of DNA and crosslinking of histones to DNA (Paabo, S., 1990). The genetic distances obtained from comparing the *Minchinia* sequences obtained from rock and pearl oyster samples were considerably less than the distance between known species for this region. In addition, the sequences obtained from the pearl oysters were not grouped together but were distributed between the rock oyster *Minchinia* sp. sequences.

The PCR results were further validated by *in situ* hybridisation with a 25 base oligonucleotide probe (SSRDb). The probe did not detect closely related haplosporidians: *Haplosporidium costale, H. nelsoni* or *Minchinia teredinis* but did produce a positive reaction from some of the haplosporidian parasites in *Haplosporidium* sp.-infected pearl oyster spat. Overall, three separate variable sections of the SSU region of the parasite's rRNA gene were tested by two different molecular methods and found to be consistent with the *Minchinia* sp. detected in rock oysters.

It is possible that the two haplosporidian species detected in *Haplosporidium* sp. infected pearl oyster spat are actually the same species. Some evidence is provided for this hypothesis by the SSR69 probe reacting to all haplosporidian parasite cells in the infected pearl oysters. Inconsistencies observed between the number of histologically positive and PCR positive may be the result of fixation since the length of time samples are fixed is known to affect the efficiency of molecular assays (Diggles, B.K. *et al.*, 2003). However, this hypothesis appears unlikely. The SSRDb ISH assay did not hybridise to all haplosporidian cells present in the infected sections. In addition, Hine and Thorne (2002) compared the rock and pearl oyster parasites using TEM and noted a number of morphological differences. These differences are summarised in Table 5.3 and also make it unlikely that the two parasites are actually the same species. The most likely explanation for the positive PCR results and partial positives from *in-situ* hybridisation with the SSRDb probe is *Minchinia* sp. earlier detected in rock oysters is present in the *Haplosporidium* sp. infected pearl oyster spat. This would mean the SSR69 probe cross reacts to the *Haplosporidium* sp. in pearl oysters despite not reacting to any other haplosporidian species tested. An SEM

and TEM comparison of the pearl oyster and rock oyster parasite's spores is required for a conclusive comparison of spore ornamentation. This would also allow a taxonomic assessment of each parasite to determine if it belongs in *Haplosporidium* or *Minchinia*.

There has been a considerable amount of debate about the characters that separate the genera *Haplosporidium* and *Bonamia* from *Minchinia*. *Haplosporidium* spp. (and *Bonamia* spp. in which spores have been detected *i.e*. *Bonamia perspora* (Carnegie, R.B., Burreson, E.M., Hine, P.M., Stokes, N.A., Audemard, C., Bishop, M.J. and Peterson, C.H., 2006) are now largely defined as species whose spores possess tails or filaments attached to the spore wall, which are formed by the same material as the spore wall (Azevedo, C., 2001; Azevedo, C., Balseiro, P., Casal, G., Gestal, C., Aranguren, R., Stokes, N.A., Carnegie, R.B., Novoa, B., Burreson, E.M. and Figueras, A.J., 2006; Azevedo, C. *et al.*, 2003; Burreson, E.M., 2001; Hine, P.M. and Thorne, T., 1998). On the other hand, the genus *Minchinia* is described as without tails (Azevedo, C., 2001) or possessing epispore cytoplasmic extensions (Azevedo, C., Balseiro, P., Casal, G., Gestal, C., Aranguren, R., Stokes, N.A., Carnegie, R.B., Novoa, B., Burreson, E.M. and Figueras, A.J., 2006; Azevedo, C., Conchas, R.F. and Montes, J., 2003; Burreson, E.M., 2001; Hine, P.M. and Thorne, T., 1998) that disappear during the maturation process (Azevedo, C., Balseiro, P., Casal, G., Gestal, C., Aranguren, R., Stokes, N.A., Carnegie, R.B., Novoa, B., Burreson, E.M. and Figueras, A.J., 2006; Azevedo, C., Conchas, R.F. and Montes, J., 2003; Azevedo, C. *et al.*, 1999). Currently, phylogenetic analysis supports the morphological characters used to define *Minchinia* with the genus being monophyletic with a bootstrap support of 58% (Chapter 3). Based on the comparison of the SSU rRNA sequences obtained in this

study with the SSU rRNA gene obtained from the putative *Minchinia* sp. in rock oysters one would expect the spores of this parasite to be without spore wall tails or possessing epispore cytoplasmic extensions (ECEs) which may disappear during the maturation process. Since *Haplosporidium* sp. has filaments composed of spore wall material, the most likely explanation for the positive PCR results and partial positives from *in-situ* hybridisation is the *Minchinia* species earlier detected in rock oysters, is present in the *Haplosporidium* sp. infected pearl oyster spat. An SEM analysis of the rock oyster parasite's spores is required for a conclusive comparison of spore ornamentation between the two species.

The presence of co-infections of haplosporidian parasites is not unprecedented. Co-infections of *Haplosporidium nelsoni* and *Haplosporidium costale* have been previously observed in *Crassostrea virginica* on the eastern sea board of the United States (Stokes, N.A. and Burreson, E.M., 2001). Both *Haplosporidium* sp. and *Minchinia* sp. parasites appear to be opportunistically parasitising weakened pearl oyster spat. Both parasites have only been detected in juvenile pearl oysters less than 12 mm in size. It appears likely that as the pearl oyster increases beyond this size it may be able to respond effectively to the parasites. Another haplosporidian parasite has been documented infecting pearl oysters in a similar fashion as the *Minchinia* sp. and *Haplosporidium* sp. There have been sporadic reports of *Bonamia roughleyi* like infections of pearl oysters (Humphrey, J.D., Norton, J.H., Jones, J.B., Barton, M.A., Connell, M.T., Shelley, C.C. and Creeper, J.H., 1998; SCFH, 1993). In the mid 1990s a *Bonamia roughleyi* like organism was found infecting pearl oyster spat in Carnarvon, Western Australia (Norris, R., 1996).

The uni-nucleated naked cells identified as the origin of the *Minchinia* sequence in Chapter 3 appear similar to a *Bonamia* species. The *Minchinia* are a sister taxon to the *Bonamia* and consequently the possible dominance of a uni-nucleated cell stage in the *Minchinia* sp. infected rock oysters described in Chapter 3 may be a result of an evolutionary relationship between these two genera. Also, there appears to be little information in the literature on the non-spore life stages of most *Minchinia* species; rather most authors report only spore morphology (Azevedo, C., 2001; Ball, S.J., 1980; McGovern, E.R. and Burreson, E.M., 1990). If the cryptic uni-nucleated stages detected in Chapter 3 are a dominant part of the life-cycle of the *Minchinia* then this may explain this lack of information. In addition, it is possible the previously described *Bonamia* infections in pearl oyster spat are actually *Minchinia* sp. infections. *Bonamia* infected pearl oyster spat are required for further analysis.

Mass mortalities of juvenile pearl oysters are well recognised (Humphrey, J.D., Norton, J.H., Jones, J.B., Barton, M.A., Connell, M.T., Shelley, C.C. and Creeper, J.H., 1998). It is possible that pearl oysters below certain sizes are susceptible to infection by parasites that would otherwise be considered too host specific to be a threat. Indeed, there is a range of potentially serious pathogens in wild bivalves that juvenile pearl oysters are exposed to upon deployment (Hine, P.M. and Thorne, T., 2000). Sequential examination of batches of juvenile oysters up to 23 weeks from deployment in the Northern Territory of Australia demonstrated progressive colonisation by a range of unidentified protozoan and metazoan organisms (Humphrey, J.D., Norton, J.H., Jones, J.B., Barton, M.A., Connell, M.T., Shelley, C.C. and Creeper, J.H., 1998). This may explain some of the mortalities experienced by the pearling industry on deployment of spat to grow-out leases.

Attempts to amplify *Haplosporidium* sp. DNA using a variety of degenerate primers have so far been unsuccessful. The primers used included all combinations of the HAP primers (Renault, T., Stokes, N.A., Chollet, B., Cochennec, N., Berthe, F., Gerard, A. and Burreson, E.M., 2000), protozoan (Carnegie, R.B., Meyer, G.R., Blackbourn, J., Cochennec-Laureau, N., Berthe, F. and Bower, S.M., 2003) and others of novel design (unpublished observations;Appendix 2) so as to target smaller DNA from formalin fixed tissues. Specificity was lowered in some of these reactions but no *Haplosporidium* sp. amplifications could be obtained. It is likely problems with poor fixation combined with non-specific and degenerate primers prevented amplification. The F1B/R2B and SSF66/SSR69 primers were specifically designed to target *Minchinia* sp. DNA and perhaps for this reason were successful. Further research into the molecular characterisation of *Haplosporidium* sp. will require either frozen or ethanol stored samples. These samples should be obtained the next time the parasite is detected using the replicate sampling method outlined in Chapter 2.

The molecular evidence provided in this chapter gave some evidence to suggest the *Minchinia* sp. detected in rock oyster is the same species as the *Haplosporidium* sp. infecting pearl oysters. The primary evidence for his hypothesis is the SSR69 probe reacting to all haplosporidian parasites in the infected pearl oyster spat. Also, all attempts to obtain a *Haplosporidium* sp. sequence from the infected pearl oysters were unsuccessful. Only *Minchinia* sp. sequences were amplified. However, there is also evidence to suggest positive PCRs are the result of a co-infection. The principle evidence for this hypothesis is the selective hybridisations obtained from the ISH assays using the SSRDb probe and the morphological differences between the two

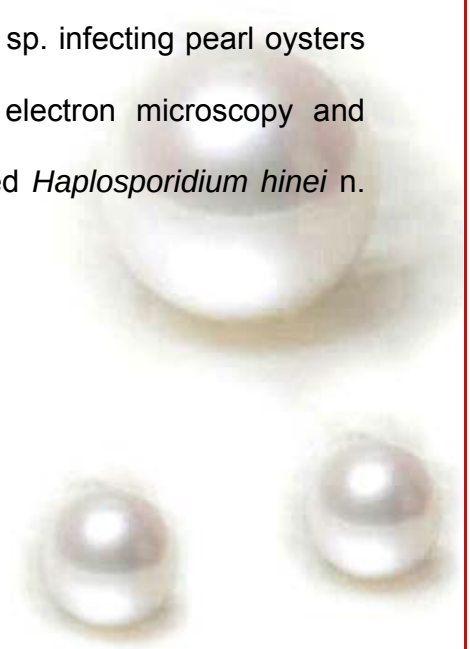
parasites highlighted by Hine and Thorne (2002) in a TEM analysis of the rock oyster parasites spores. Since attempts to obtain sequence data from the *Haplosporidium* sp. were unsuccessful, further analysis of the morphological characteristics of the rock and pearl oyster parasite spores are required to conclusively resolve this conflict.

Table 5.3 Summary of the major differences in the spores of the pearl oyster parasite and the rock oyster parasite**.**

Pearl oyster parasite (Hine and Thorne 1998)	**Rock oyster parasite (Hine and Thorne 2002)**
Elongated to ovoid 6.7 – 7.7 x 3.8-4.3	5.6 – 6.7 x 3.3 – 4.0
Wall 160 nm	Wall 250 nm
Surface filaments 62 nm	Not seen
Sporoplasm nucleus usually basal but sometimes equatorial	Nucleus equatorial
Haplosporosomes 230 nm Sometimes elongated to 410 nm	Haplosporosomes 50-67 x 330nm Ovoid to spherical 100 – 150 nm Or small spherical and dense 60-70nm
Not seen	Rod like structures developed posteriorly in maturing spores
Not seen	Lipid droplets

Chapter 6 : Spore ornamentation of *Haplosporidium hinei* n.sp. in pearl oysters *Pinctada maxima*.

In this Chapter the spore ornamentation of *Haplosporidium* sp. infecting pearl oysters is formally described using transmission and scanning electron microscopy and compared to other haplosporidians. The parasite is named *Haplosporidium hinei* n. sp.



6.1 Introduction

The morphology and origin of spore ornamentation, variously termed wrappings, extensions, filaments or tails, is the principle taxonomic feature used to distinguish species and genera within phylum Haplosporidia. However, the spore ornamentation is not known for many haplosporidian species and this makes species differentiation and genus assignment difficult (Burreson, E.M. and Reece, K.S., 2006). It is likely that the taxonomy of the Haplosporidia will remain confused until spore ornamentation is characterised for a large number of species.

Currently, the most common method to separate the genera *Haplosporidium* and *Bonamia* from the *Minchinia* is based on the origin of the spore ornamentation, from either the spore wall in the case of *Haplosporidium* and *Bonamia*, or from the epispore cytoplasm in the *Minchinia* (Azevedo, C., Montes, J. and Corral, L., 1999; Burreson, E.M., 2001; Hine, P.M. and Thorne, T., 1998; Hine, P.M. and Thorne, T., 2002; Ormieres, R., 1980). The spore ornamentation from only one species of the *Bonamia* has been characterised. That species *Bonamia perspora*, has been found to possess strap like projections derived from the spore wall (Carnegie, R.B., Burreson, E.M., Hine, P.M., Stokes, N.A., Audemard, C., Bishop, M.J. and Peterson, C.H., 2006). The emphasis on spore ornamentation in the taxonomy of the Haplosporidia means it is an important characteristic to be described.

The objective of this chapter is to formally describe the spore ornamentation of the haplosporidian in pearl oysters using both scanning and transmission electron microscopy and compare the results to other described haplosporidians and to the

molecular data obtained in the previous chapters. The pearl oyster parasite is named in this chapter as *Haplosporidium hinei* n. sp.

6.2 Methods and materials

Archived formalin fixed, paraffin embedded pearl oyster tissues infected with *Haplosporidium hinei* n. sp. were obtained from the Western Australian Department of Fisheries and were fixed whole in 10% formalin made up with seawater. This material had been collected from Carnarvon, Western Australia as part of a routine disease clearance prior to transport (Hine, P.M. and Thorne, T., 1998). Material specifically fixed for electron microscopy was not available. Sections were processed and stained for histological examination with hematoxylin and eosin. Briefly, unstained sections were cut 4 µm thick and placed on slides. Sections were dewaxed with xylene and rehydrated in an ethanol series (2 changes for 2 min each). The sections were placed in haematoxylin for 5 min, rinsed in water and then placed in Scott's tap water substitute until blue. The slides were again rinsed in water followed by 95% ethanol (1 change for 30 s). The sections were stained with 1% eosin for 30 seconds and finally dehydrated in 95% ethanol (2 changes 30 s each), absolute ethanol (2 changes 30 s each) and xylene (3 changes 30 s each). Ornamentation on the spores of *H. hinei* n. sp. was assessed through light microscopy, transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

Paraffin embedded tissue was prepared for TEM by dewaxing in xylene overnight, hydrating through three changes of 100, 90 and 70% ethanol. A 1 mm^3 cube of digestive gland tissue was dissected in a puddle of 5% Sorensen's phosphate buffered gluteraldehyde. The sections were then placed in 5% glutaraldehyde overnight. The samples were then washed in 0.07 M Sorensen's phosphate buffer.

The tissues were immersed in Dalton's Chrome Osmic Acid for 60-90 mins at 4° C. The samples were then dehydrated through an ethanol series. Following this, the tissue was immersed in Propylene Oxide, a 60: 40 Propylene oxide/Epon mixture and finally pure 'Epon' overnight. Embedded tissues were sectioned with an mU3/C (Reichert, Austria) ultramicrotome and stained with uranyl acetate. Analysis was performed with a Philips CM100 Bio TEM.

For SEM, infected oyster tissue was removed from the paraffin block and deparaffinised in xylene for three days. The tissue was rehydrated through a graded series of ethanol (100%, 90%, 70%, 50% and 30%). The tissue was then sonicated in pure water. A puddle of the resulting suspension was placed onto six 12 mm diameter coverglasses coated with poly-L-lysine; spores were allowed to settle for 1 h in a moist chamber. The coverglasses were then dipped in water to remove the excess material and dehydrated through an ethanol series. The affixed spores were then subjected to critical point drying in liquid CO_2 and coating with gold:palladium. Characterisation of the spore ornamentation was based on observations of 26 spores with a Zeiss Leo 435vp.

6.3 Results

6.3.1 Light microscopy

Light microscopy of the sections revealed large numbers of presporulation and sporulation stages of the parasite in the connective tissue surrounding the digestive gland and within the digestive diverticulae (Figure 6.1). Mature spores with a yellow refractile wall enclosing an eosinophilic sporoplasm were observed. Spore ornamentation was not apparent with light microscopy and parasites were not observed in the epithelia.

6.3.2 Electron microscopy

Although the fixative used for the study was suboptimal for electron microscopy, it was sufficient to permit observation of the spore wall and its ornamentation. The fixed spores of *H. hinei* n. sp. were pleomorphic, or elongated 3.5 µm – 4 µm x 2.5 µm – 3.0 µm in size (Figure 6.2). The sporoplasm nucleus was usually basal, but sometimes equatorial and measured 0.8 µm – 1.3 µm in diameter (mean= 1.03 µm). The operculum was situated in the apical zone of the wall and consisted of a circular lid of 1.9 µm to 2.6 µm (mean= 2.24 µm) diameter (Figure 6.2). The lid of the operculum was about 7.2 nm thick and was connected to the spore wall by a hinge. Microfilaments 45 nm – 75 nm (mean= 60.72 nm) long and 28.5 – 34.3 nm (mean= 31.25 nm) wide were also observed on the surface of the spore wall (Figure 6.2). The microfilaments were composed of spore wall material and had focal distribution but were not confined to any particular section of spore wall. They were observed in both immature and mature spore stages.

Round or elongated haplosporosomes were present that varied in diameter from 90 nm to 500 nm (mean= 256 nm; Figure 6.2). Many of the haplosporosomes contained an internal membrane that was either circular or shaped as an axe head (Fig 6.2). In the apical zone of the endosporoplasm, a spherule was formed by several vesicles approximately 0.1 µm in diameter (Figure 6.2). Bundles of microfilaments were also occasionally observed in the spore endosporoplasm (data not shown).

Obvious by both TEM and SEM were two filaments that were wound around the spore which appeared to originate from two posterior spore wall thickenings that resembled "knobs" (Figures 6.2 & 6.3). The thickenings seemed to appear late in the development of the spore and were approximately 700 nm long. Both filaments passed up one side of the spore together until just below the operculum where upon the filaments split and passed obliquely under each side of the lip of the opercula lid (Figure 6.3). There was one filament on each side of the spore (Figure 6.3). Both filaments wrapped around the spore four or five times and decreased in diameter toward the distal end (Figure 6.2). The number and arrangement of the filaments was confirmed by TEM. Each filament was approximately 27 µm in length. The filaments were derived from the spore wall and were not projections of the epispore cytoplasm. A second set of branching tubular filaments composed of a different material was observed on the spore body although not on mature spores possessing a "knob-like" posterior thickening (Figure 6.2)

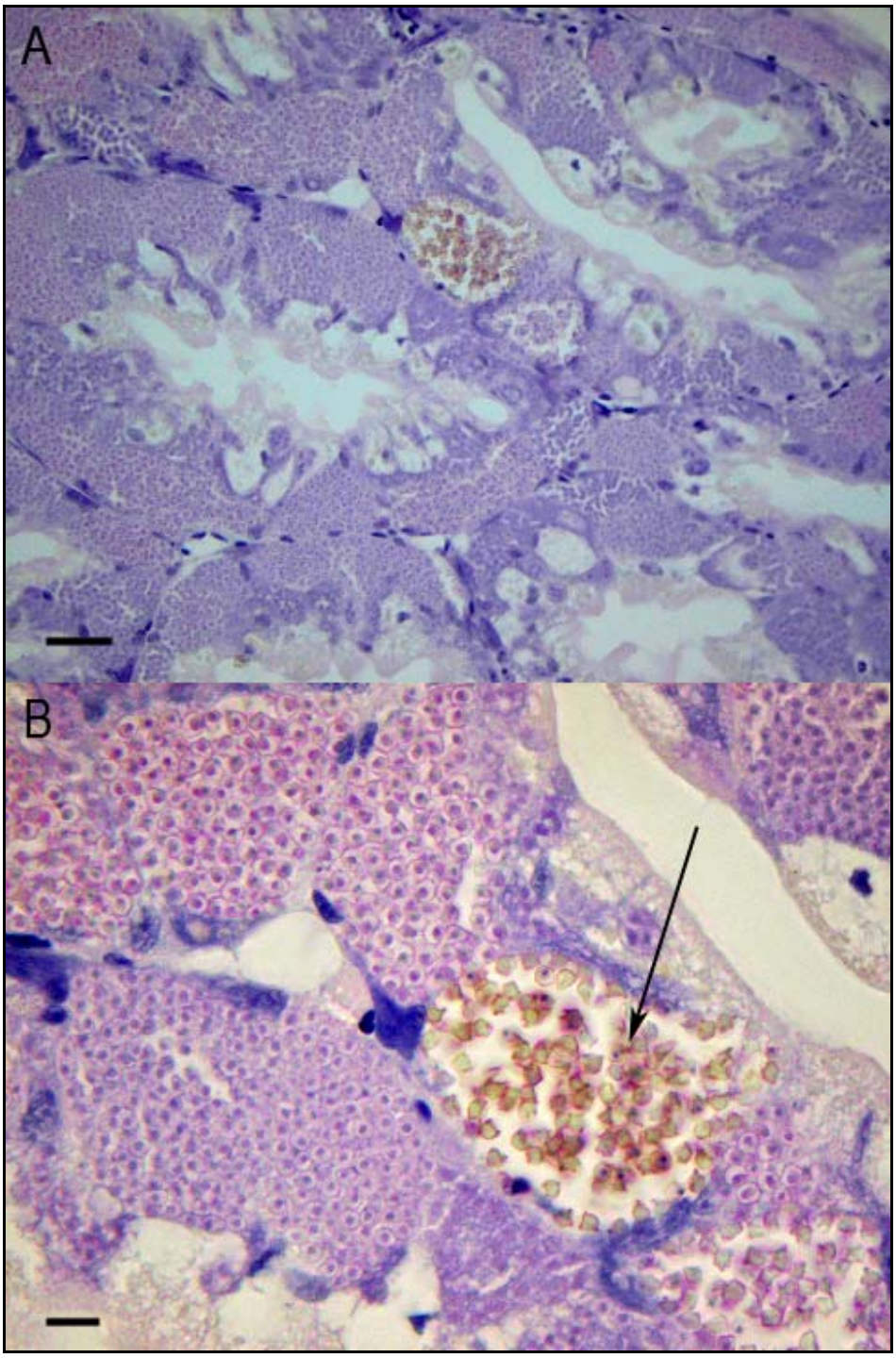


Figure 6.1 Pearl oyster digestive gland containing large numbers of the presporulation and sporulation stages of *Haplosporidium hinei* n. sp. (A) Low magnification view of the digestive gland. Scale bar = 30 µm. (B) Higher magnification view of the same section including the mature spores. Arrow indicates the yellow refractile mature spores. Scale bar = 15 µm.

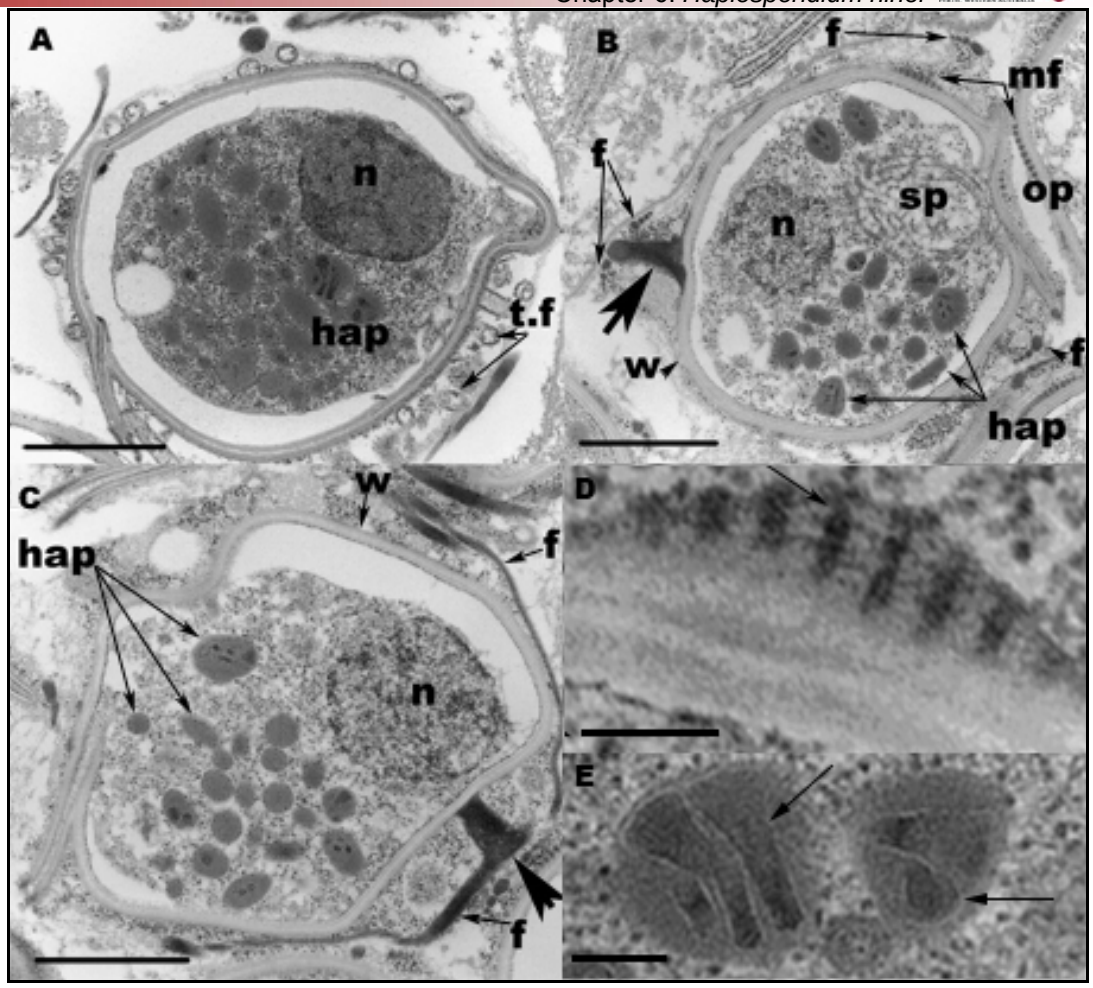


Figure 6.2 Transmission electron micrographs (TEM) of mature spores of *Haplosporidium hinei* n.sp. infecting the pearl oyster *Pinctada maxima*. Haplosporosomes (hap) with internal membranes are also present in each photograph. n refers to the basal nucleus, w refers to spore wall, sp refers to the spherule, op refers to the operculum. (A) Indicates the tubular filaments (tf). Tubular filaments are not present in mature spores possessing a posterior thickening (see 2-3 below). Scale bar = 1.5 µm. (B) Shows the posterior thickening and attached spore wall filaments (f) in cross section. Scale bar = 1.5 µm. (C) Indicates the spore wall filaments (f) and posterior knob in longitudinal section. Scale bar = 1.5 µm. (D) A higher magnification view of the microfilaments (arrow). Scale bar = 240 nm. (E) A higher magnification view of the haplosporosomes containing the axe shaped internal membrane structures. Scale bar = 200 nm.

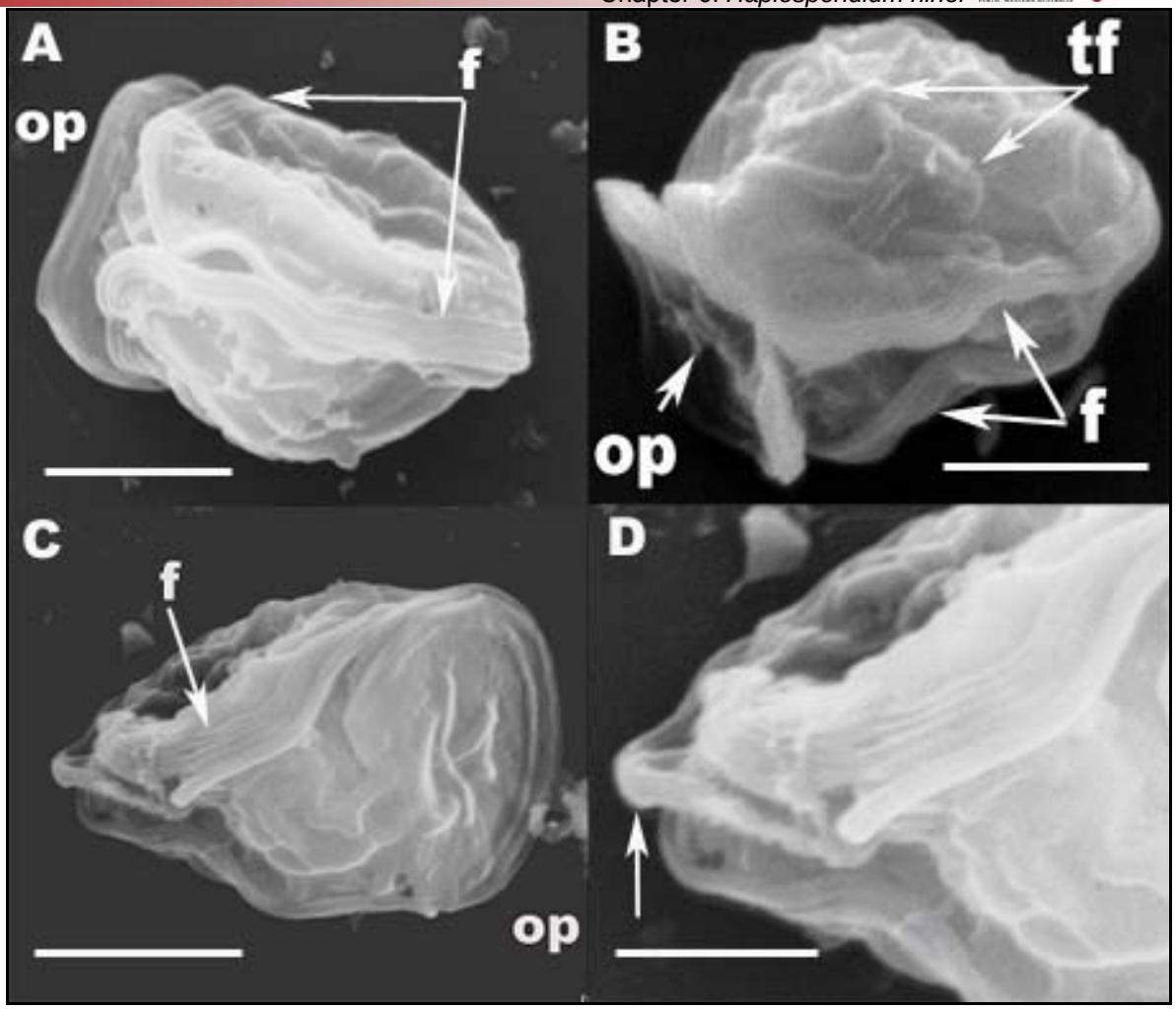


Figure 6.3 Scanning electron micrographs indicating the spore ornamentation of *Haplosporidium hinei* n. sp. infecting *Pinctada maxima* in supposed order of development. (A) Spore showing a filament (f) wrapping each side of the spore to the operculum (op). Scale bar = 1.5 µm (B). Spore showing the tubular filaments (tf) and spore wall filaments (f). Scale bar = 1.5 µm. (C) Spore showing the origin of the filaments (f) at the posterior knob-like thickening (large arrow). Scale bar = 1.5 µm. (D) Higher magnification view of the basal section of the spore with the posterior knob-like thickening (large arrow). Scale bar = 0.8 µm.

6.3.3 Taxonomic summary

Haplosporidium hinei Bearham, Spiers, Raidal, Jones, Burreson and Nicholls n. sp.

Description and identifying characteristics

Two spore wall derived filaments that are wound around the spore and originate from a posterior spore wall thickening. Both filaments pass up one side of the spore together until just below the operculum where upon the filaments split and pass obliquely under each side of the lip of the opercula lid. There is one filament on each side of the spore. Both filaments are wrapped around the spore four or five times and decrease in diameter toward the distal end. Each filament is approximately 27 µm in length.

Type Host

Pinctada maxima (Mollusca, Bivalva, Pteriidae).

Site of infection

Digestive gland epithelium, gills and mantle.

Type locality

Carnarvon (latitude: 24° 53' S, longitude: 113° 40' E), Western Australia.

Material deposited:

H&E slides at the Western Australian Museum.

Etymology

The epithet refers to Dr Mike Hine who has contributed a significant amount to our understanding of the haplosporidians and who originally described this parasite.

6.4 Discussion

The parasite described in this study shows the typical morphology, spore structure and spore ornamentation of a haplosporidian species. The internal organisation of the spore endosporoplasm shows a similar arrangement to other haplosporidian species. This was characterised by the presence of a basal or equatorial nucleus, an apical spherule and several electron dense membrane bound haplosporosomes. The presence of an orifice which is covered with an operculum suggests the parasite belongs to either *Minchinia*, *Haplosporidium* or *Bonamia*.

If the criteria proposed by Ormieres (1980) for distinguishing the genera *Haplosporidium* and *Minchinia* are followed then *Haplosporidium hinei* n.sp was correctly assigned to *Haplosporidium* by Hine and Thorne (1998) because the filaments are derived from the spore wall and not from epispore cytoplasm. The similarity of *H .hinei* to *H. lusitanicum*, *H. pickfordi, H. montiforti*, *H. parisi* and *H. comatulae* in the origin of the ornamentation from the basal position on the spore justifies its placement within *Haplosporidium*. The alternative genus with ornamentation originating from the spore wall is *Bonamia*. The sole member of the *Bonamia* that has been found to possess spores, *Bonamia perspora* has strap like projections which do not originate from a basal position on the spore (Carnegie, R.B., Burreson, E.M., Hine, P.M., Stokes, N.A., Audemard, C., Bishop, M.J. and Peterson, C.H., 2006).

When compared to Hine and Thorne (1998), the fixed spores in the current study, at 3.5 µm – 4 µm x 2.5 µm – 3.0 µm (Figure 6.2) are smaller than those described previously and Hine and Thorne (1998) also did not observe posterior knobs or

surface filaments in their TEM study. Otherwise, the microfilaments and haplosporosomes observed here are consistent with those described previously.

The ornamentation on the spores of the pearl oyster parasite described by the number of filaments, their length, insertion points on the wall and organisation of the filaments is unique among described haplosporidian species where spore ornamentation is known. *Haplosporidium armoricanum* from *Ostrea edulis* in Europe has paired filaments arising from each end of the spore (Azevedo, C., Montes, J. and Corral, L., 1999). *Haplosporidium nelsoni* and *H. costale* from *Crassostrea virginica* along the east coast of the United States do not have paired filaments arising from the posterior end of the spore; rather they have wrappings around the spore (Burreson, E.M. and Reece, K.S., 2006). *Haplosporidium edule* from cockles in Europe has many knobbed extensions of the spore wall, not long filaments (Azevedo, C., Conchas, R.F. and Montes, J., 2003).

The basal origin of the filaments described in this study is similar to that of the spores occurring in the gastropod haplosporidians of *Haplosporidium montforti, H. pickfordi* and *H. lusitanicum.* These species have long, spore wall derived filaments that originate at the basal end of the spore. However, these parasites lacked the distinctive axe shaped internal membranes observed within the haplosporosomes of the pearl oyster parasite. In addition, *Haplosporidium montforti* described infecting the abalone *Haliotis tuberculata* in Spain, has four filaments, 20-28 µm long, with two attached opposite each other at the basal end and two other opposing filaments attached at the apical end of the spore (Azevedo, C., Balseiro, P., Casal, G., Gestal, C., Aranguren, R., Stokes, N.A., Carnegie, R.B., Novoa, B., Burreson, E.M. and

Figueras, A.J., 2006). Also, the filaments of the pearl oyster parasite are round in transverse section rather than the L,T or X-like sections of *H. montfordi* (Azevedo, C., Balseiro, P., Casal, G., Gestal, C., Aranguren, R., Stokes, N.A., Carnegie, R.B., Novoa, B., Burreson, E.M. and Figueras, A.J., 2006). *Haplosporidium pickfordi* from fresh water snails in the great lakes region of the United States, has two posterior knob-like thickenings like the pearl oyster parasite but has approximately nine filaments wound around the spore rather than the two filaments observed here (Burreson, E.M., 2001). While *H. lusitanicum* described from the European gastropod *Helcion pellucidus*, has two basal opposing filaments ~112 µm long, and coiled around the spore 10-13 times which are elliptical in transverse section (Azevedo, C., 1984).

The basal origin, arrangement of the spore wall filaments and the surface microfilaments is most reminiscent of *Haplosporidium parisi* among known species. In *H. parisi* filaments are also wound around the spore and pass obliquely under the operculum. The filaments in *H. parisi* are also circular in transverse section (unlike *H. lusitanicum*). Ormieres (1980) also described the presence of small bumps on the spore wall of *H. parisi* which appear to be similar to the microfilaments described in this study except the “bumps” in *H. hinei* are closer together and are more focally distributed compared to *H. parisi* (Figure 6.2). *Haplosporidium hinei* n. sp. differs from *H. parisi* in the length of filaments and the presence of branching tubular filaments on the surface of the spores (Ormieres, R., 1980). Filaments in *H. parisi* were up to 300 µm long and were wrapped around the spore approximately 18 times while in the pearl oyster parasite the filaments were wrapped around the spore only four or five times and are considerably shorter at approximately 27 µm.

A similar situation occurs with *Haplosporidium comatulae* an endoparasite of the crinoid echinoderm *Oligometra serripinna* in north eastern Australia which also possesses spore wall filaments with a similar arrangement to *H. parisi* and *H. hinei*. Filaments in *H. comatulae* are round in cross section but may be longer than *H. parisi* since they wrap the spore approximately 32 times (La Haye, C.A. *et al.*, 1984). La Haye et al (1984) did not observe any internal structures within the haplosporosomes of *H. comatulae* or microfilaments on the spore wall.

The definition of *Haplosporidium* has been confounded by a lack of knowledge of the spore ornamentation of the type species *Haplosporidium scolopli* Caullery and Mesnil. Like *H. scolopli*, *H. parisi* is a parasite of serpulid polychaetes of the French Atlantic coast. Ormieres (1980) concluded *H. parisi* to be very close to *H. scolopli* and consequently, the pearl oyster parasite may also be morphologically similar to *H. scolopli.*

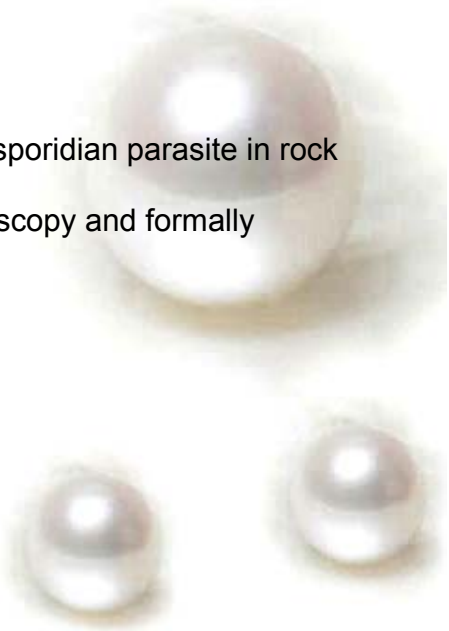
Speculatively, the function of the spore wall filaments may be to aid in floatation. The filaments may be extended once the spore is released to the environment. Characterisation of spore ornamentation requires both TEM and SEM examination. This has not been achieved for many haplosporidian species and it will probably require DNA sequence analyses of a large number of species in the phylum to elucidate the relationships among the different morphological types. Further study is required to clarify the relationship between the parasite described here and the haplosporidian species infecting rock oysters on the same coast. The rock oyster

parasite described by Hine and Thorne (2002) also possessed a posterior thickening with filaments attached in < 2% of spores.

Chapter 5 provided evidence that the pearl oyster and rock oyster parasite were the same species and Chapter 3 suggested the parasite was a *Minchinia* species. The results obtained in this chapter clearly show the pearl oyster parasite is a *Haplosporidium* spp. It is possible the SSR69 probe developed in this study might cross-react to the *Haplosporidium hinei* n. sp despite assessment of its specificity against six closely related haplosporidians in Chapter 2. It is impossible to rule out a cross reaction with an as yet undescribed parasite. An alternative explanation is the presence of a *Minchinia* spp. with *Haplosporidium* spore morphology. This would suggest *Minchinia* is paraphyletic. To date only three *Minchinia* spp. have been described both morphologically and by sequence analysis. Indeed, it appears the *Bonamia* which group with the *Minchinia* in phylogenetic analyses (Figure 3.5) possess a *Haplosporidium* spore morphology with the presence of spore wall ornamentation in the spores of *Bonamia perspora* (Carnegie, R.B., Burreson, E.M., Hine, P.M., Stokes, N.A., Audemard, C., Bishop, M.J. and Peterson, C.H., 2006). Both the rock oyster and pearl oyster parasites were originally described as *Haplosporidium* species. In addition, the rock oyster parasite described by Hine and Thorne (2002) also possessed a posterior thickening with filaments attached in < 2% of spores. An SEM and TEM analysis of the spores of the rock oyster parasite is required so that its ornamentation can be compared to the spores of *Haplosporidium hinei* n. sp. described here.

Chapter 7 : Spore ornamentation of *Minchinia occulta* n. sp. in rock oysters *Saccostrea cucullata* (Born, 1778).

This chapter describes the spore ornamentation of the haplosporidian parasite in rock oysters using both transmission and scanning electron microscopy and formally names the parasite *Minchinia occulta* n. sp.



7.1 Introduction

Mortalities (up to 80%) among rock oysters *Saccostrea cucullata* were first recognised by energy companies operating on the North West Gas Shelf of Western Australia in the early 1990s (Hine, P.M. and Thorne, T., 2002). The companies submitted samples for diagnosis and a *Minchinia* species (Haplosporidia: Haplosporidiidae) parasitising rock oysters was subsequently morphologically described by Hine and Thorne (2002) using light and transmission electron microscopy.

The parasite was not able to be diagnosed following the development of a specific probe for *Haplosporidium nelsoni* despite several attempts (Hine, P.M. and Thorne, T., 2002). However, a section of the organism's rRNA gene was sequenced and an *in situ* hybridisation assay was developed in Chapter 3. The parasite fell within the *Minchinia* in a phylogenetic analysis of the parasite's SSU rRNA gene and is a sister taxon to a clade composed of *M. chitonis*, *M. teredinis* and *Minchinia* sp.

Currently, species and genera within phylum Haplosporidia are differentiated on the basis of the origin and morphology of spore ornamentation. However, species identification and genus assignment has been confounded since spore ornamentation is not known for many haplosporidian species (Burreson, E.M. and Reece, K.S., 2006). Indeed, only three species of *Minchinia* have been described by both electron microscopy and molecular analysis. The taxonomy of the Haplosporidia may remain confused until spore ornamentation is characterised for a large number of species. It may also require DNA sequence analyses of a large number of species in the phylum to determine the relationships among the different morphological types. The use of

spore ornamentation in the taxonomy of the Haplosporidia means it is an important characteristic to be described.

This study has provided some molecular evidence suggesting the pearl oyster and rock oyster haplosporidians may be the same species. However, the selective hybridisations observed in the SSRDb ISH assay suggest the pearl oysters are infected with two parasite species. This explanation also explains the positive PCR results and appartent morphological differences between the spores of the rock and pearl oyster parasites.

The spores of both parasites as described by Hine and Thorne (1998 and 2002) do share some similarities. Both species were originally described as *Haplosporidium* spp (Hine, P.M. and Thorne, T., 1998; Hine, P.M. and Thorne, T., 2002) and Hine and Thorne (2002) Figure 14 indicates the rock oyster parasite has a posterior thickening with associated filaments. In the previous chapter, an analysis of the spore morphology of the pearl oyster parasite was undertaken and indicated a *Haplosporidium* species with a posterior thickening with two associated filaments.

This aim of this chapter is to describe the spore ornamentation of the haplosporidian parasite of rock oysters (*Saccostrea cucullata*) using both scanning and transmission electron microscopy to assess whether the parasite possesses any of the characteristics of a *Minchinia* parasite and to compare it to *Haplosporidium hinei*.

7.2 Materials and Methods

Histologically positive formalin fixed rock oysters infected with *Minchinia occulta* n. sp were obtained from the Western Australian Department of Fisheries and processed using conventional histological techniques. All of the material was collected from the same batch of infected oysters from Varanus Island on the north coast of Western Australia (co-ordinates 20°39′3″ S 115°34′27″ E).

7.2.1 In-situ hybridisation

In-situ hybridisation (ISH) was performed to confirm the identity of the parasite as the same species sequenced in Chapter 3. An oligonucleotide probe was used for this purpose. The assay was performed using the SSRDb probe using the methods outlined in Chapters 3 and 4. The presence and location of *Minchinia occulta* n.sp was confirmed using hematoxylin – eosin (H/E) stained serial sections (Figure 7.1).

7.2.2 Electron microscopy

Spores from the same batch of oysters that underwent *in-situ* hybridisation were processed for electron microscopy. Material specifically fixed for electron microscopy was not available. Ornamentation on the spores of *M. occulta* n. sp. was assessed through light microscopy, transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Sections were processed and stained for histological examination using the method outlined in Chapter 6. Measurements of 16 parasites are given in micrometers with the range in parentheses.

7.3 Results

7.3.1 Light microscopy

Histopathological examination of 16 positive oysters from Varanus Island, Western Australia revealed large numbers of parasites present usually as focal lesions in the connective tissue of the gills or disseminated around the connective tissue between the digestive diverticulae, and in the mantle (Figures 7.1 and 7.2). Large numbers of parasites were also present in the reproductive follicles of the host of some samples (Figure 7.1). All parasite stages including uni-nucleate and bi-nucleate naked cells were extra cellular. Uni-nucleate cells were 4.5 to 5.8 µm (mean = 5.2 µm) in diameter with a generally central nucleus. Multi-nucleate plasmodia with between 2 to 24 nuclei were also present in infected oysters. Mature spores with a yellow refractile wall enclosing an eosinophilic sporoplasm were observed around the digestive diverticulae (Figure 7.2). Spore ornamentation was not apparent with light microscopy and parasites were not observed in the epithelial tissue. Phagocytosis of parasites was not observed

7.3.2 In-situ Hybridisation

Parasite identity was confirmed using *in-situ* hybridisation. The SSRDb probe produced strong hybridisation signals with little background staining (Figure 7.1). The signal was not reproduced in tissues produced from uninfected oysters or in negative control material (Figure 7.1). Sections of *Minchinia teredinis*, *Haplosporidium nelsoni* and *H. costale* included in the assay did not produce a positive signal (Figure 7.3).

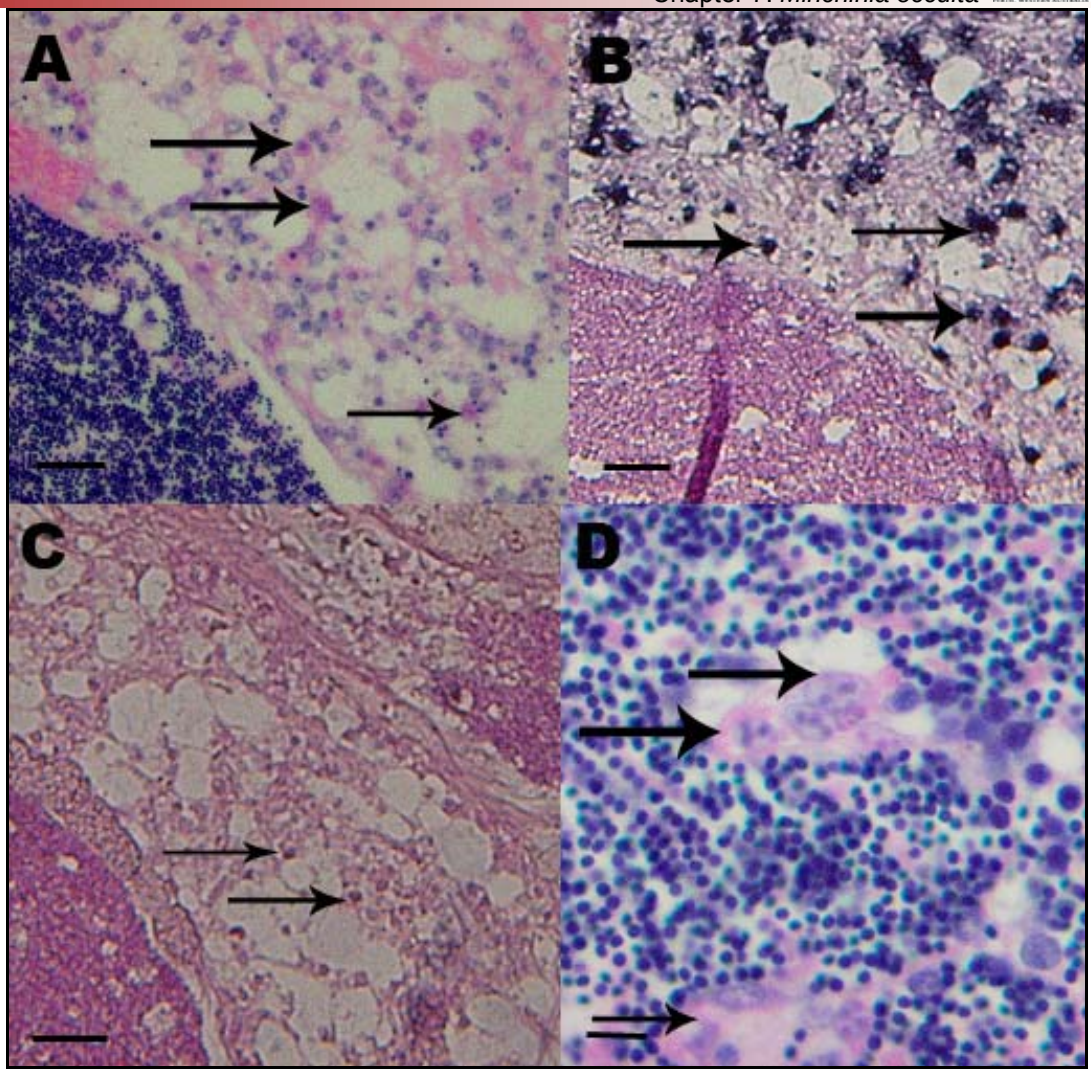


Figure 7.1 Rock oyster connective tissue and reproductive tissue containing single, bi and tri nucleate haplosporidian parasites. (A) Haematoxylin-eosin stained section containing the parasites (arrows). (B) Serial section containing the parasites in an *in-situ* hybridisation. Parasites are identified by a darker colouration. Examples are indicated with an arrow. (C) Negative control serial section from the same hybridisation. (D) Haematoxylin-eosin stained section containing the parasites in reproductive tissue of the host. Scale bars= 15 µm. All samples were taken from the same batch of infected oysters.

The fixative used for the samples was suboptimal for electron microscopy, nonetheless, it was adequate to permit observation of the spore wall and its ornamentation.

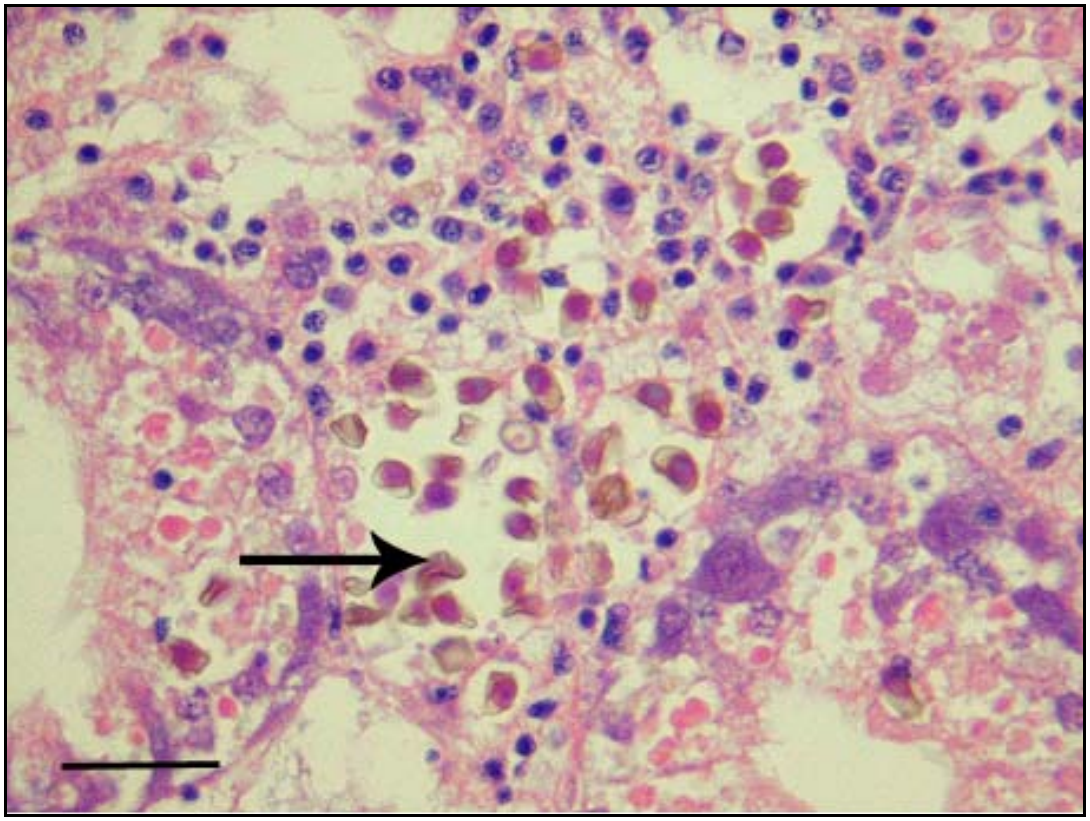


Figure 7.2 Rock oyster digestive gland containing large numbers of the presporulation and sporulation stages of *Minchinia occulta* n. sp. in a hematoxylin-eosin stained section. Arrow indicates an example of the refractile mature spores. Scale bar = 20 µm.

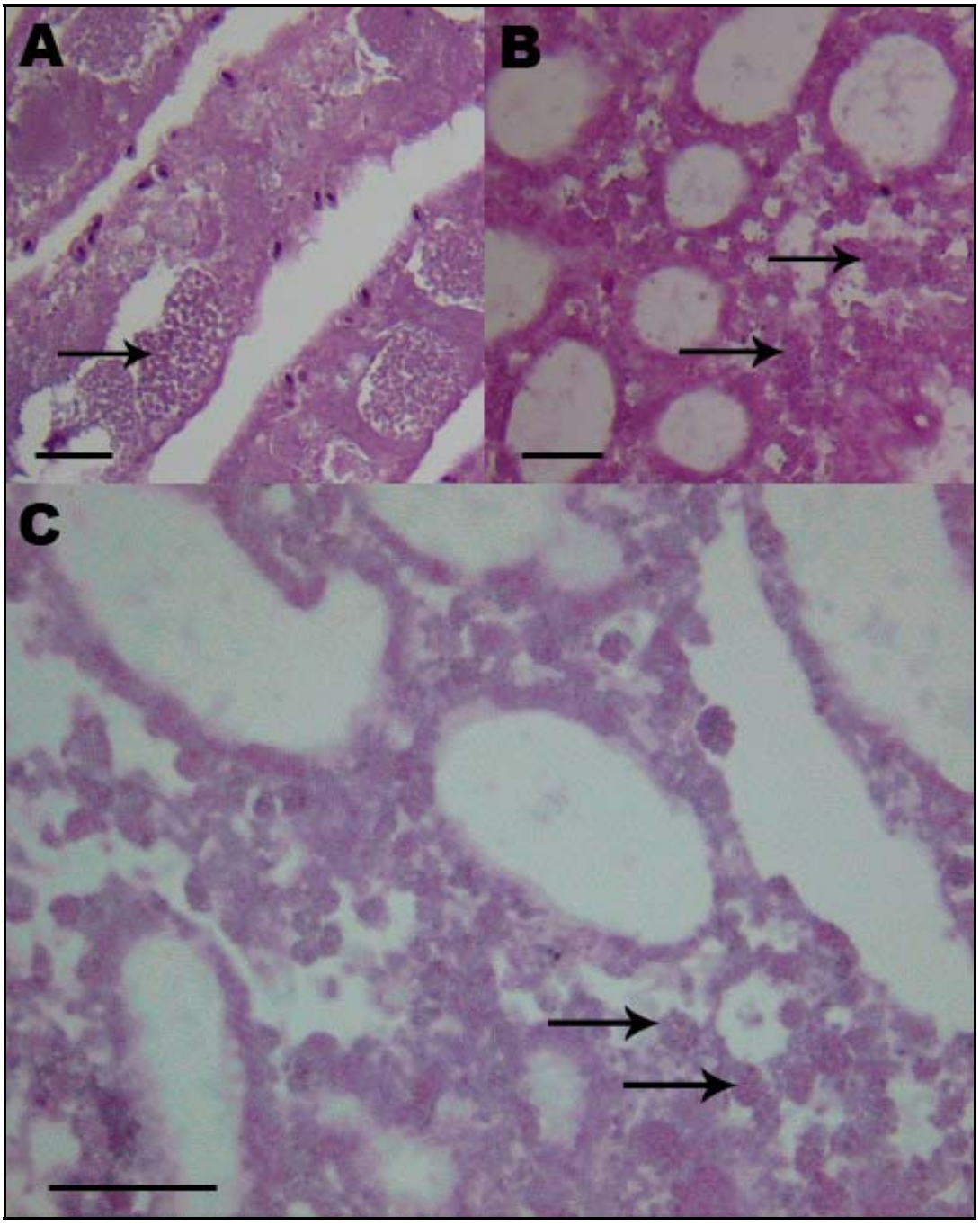


Figure 7.3 Haplosporidian parasites used to assess the specificity of the SSRDb ISH assay. Example parasites are indicated with an arrow. (A) *Minchinia teredinis* in the gills of the shipworm *Teredo* sp. Scale Bar = 10 µm. (B) *Haplosporidium costale* in an Eastern Oyster. Scale Bar= 30 µm (C) *Haplosporidium nelson*i in an Eastern Oyster. Scale bar= 20 µm. Sections are counterstained in a brazilin haematoxylin.

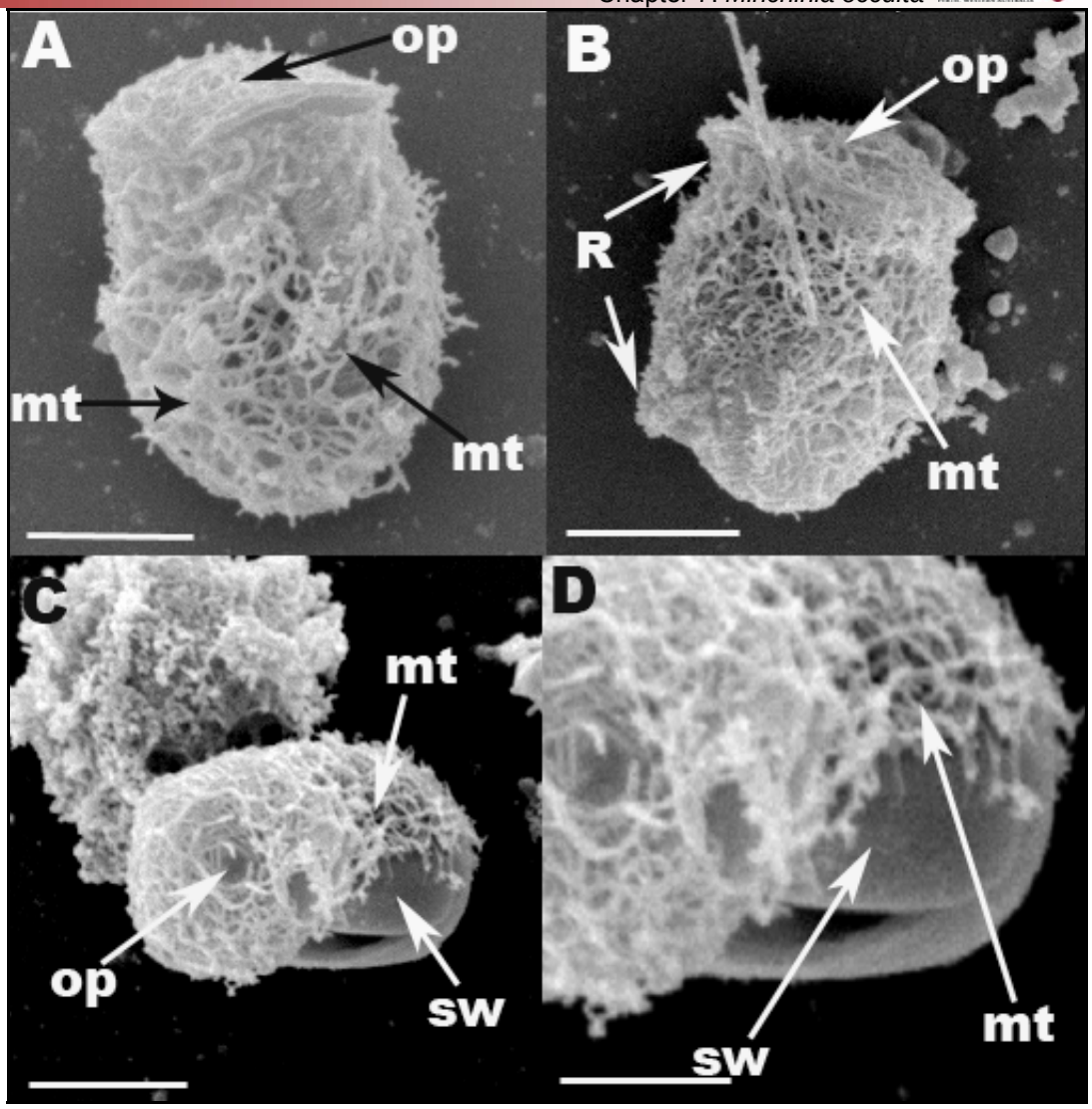


Figure 7.4 Scanning electron micrographs indicating the spore ornamentation of *Minchinia occulta* n. sp. infecting *Saccostrea cucullata*. (A) *Minchinia occulta* spore with a branching network of microtubules-like structures and opercula lid (op). Scale bar = 1.5 µm (B) Spore with a ridge running along the front of the spore (arrow). Scale bar = 2.5 µm (C) Spore with network of branching microtubule–like structures that has been partially removed or degenerated revealing a smooth spore wall (sw) beneath. Some artifact also appears on the spore wall. Scale bar = 3.0 µm. (D) Higher magnification view of the degraded microtubule like structures with exposed spore wall beneath. Scale bar = 1 µm.

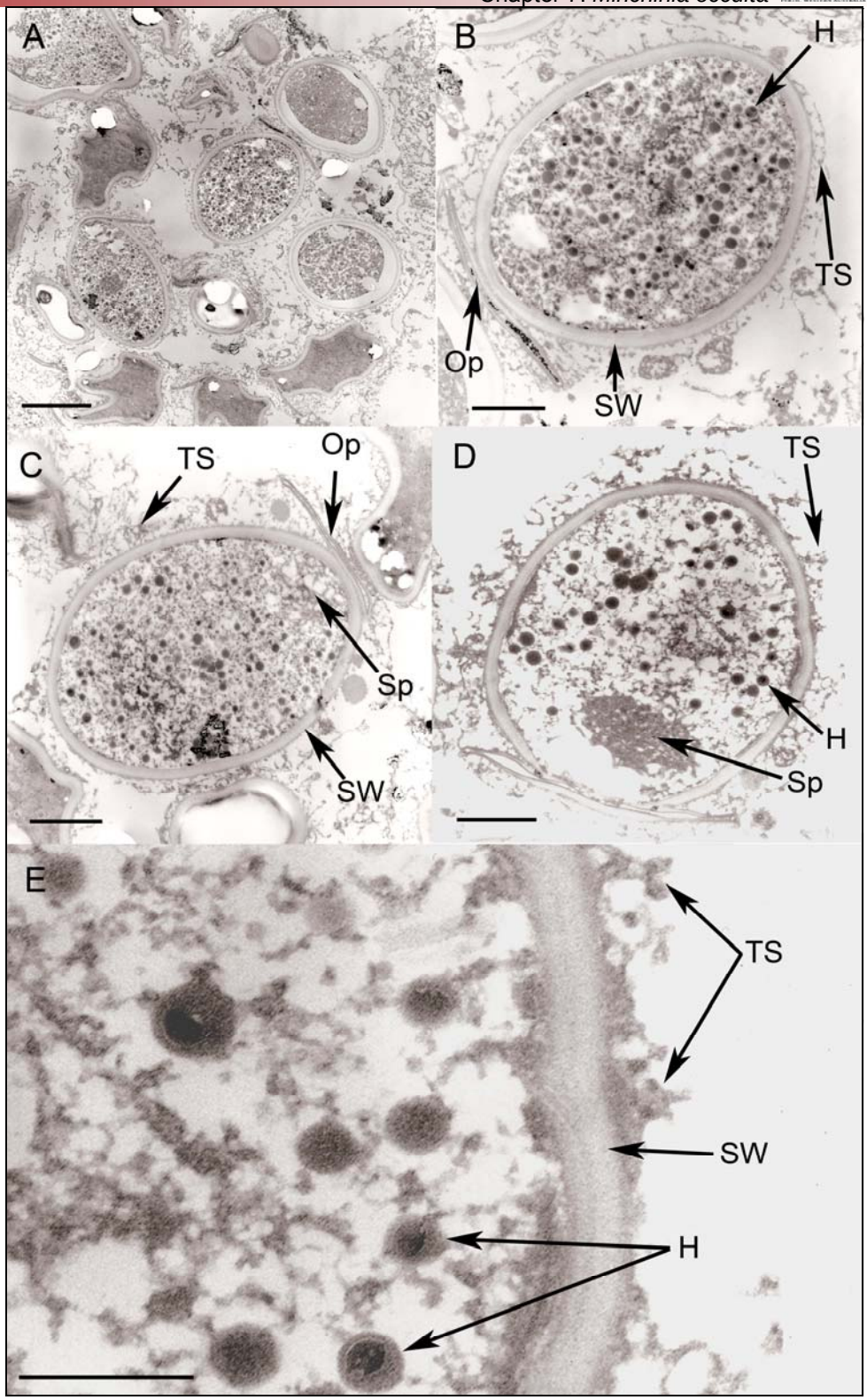


Figure 7.5 Transmission electron micrographs (TEM) of spores from *Minchinia occulta* n. sp. infecting the rock oyster (*Saccostrea cucullata*). (A) Group of spores at a variety of different spore stages none of which possess any spore wall material. Scale bar = 2 µm. (B-D) *Minchinia occulta* spores with operculum (Op), haplosporosomes containing a membrane bound body (H), Microtubular-like structures (TS) and Spore wall (SW) and spherule (Sp). Scale bars = 1 µm. (E) Higher magnification view of the membrane bound body within the haplosporosomes (H), the spore wall (SW) and the Microtubular-like structures (TS). Scale bar = 500 nm.

7.3.3 Electron microscopy

The spores of the rock oyster parasite appeared to have a network of branching microtubule-like structures covering the entire spore including the opercula lid (Figure 7.4). The microtubule-like structures varied in size but were approximately 15-30 nm (mean = 20 nm) in diameter (Figure 7.4). In some of the spores this network appeared to degrade as the spore aged revealing a smooth spore wall (Figure 7.4). A ridge was also present on one side of the spore. It began just below the operculum and ended approximately 2 µm from the aboral end of the spore (Figure 7.4). The ridge did not consist of spore wall material but appeared to be composed of the same material as the microtubule-like structures. Indeed, no spore wall derived ornamentation was observed on any spores.

The fixed spores of *M. occulta* n. sp were elongated 4.4 – 5.0 x 3.5 – 4.1 µm in size (Figure 7.4 and 7.5). The spore wall appeared to be 250 nm in thickness and consisted of 3 layers; an inner dense layer of approximately 90 nm, a middle layer of 30 nm and an outer dense layer of 130 nm (Figure 7.5). The sporoplasm nucleus was positioned equatorially and usually round in shape and measured 1.5 – 2.3 µm in diameter (mean = 1.9 µm). The operculum was situated in the apical zone of the wall and consisted of a circular lid of 2.5 – 3.0 µm (mean = 2.8 µm) diameter (Figure 7.5). The lid of the operculum was about 150 nm thick and was connected to the spore wall by a hinge.

Round electron dense round (150 nm - 180 nm (mean = 160 nm) in diameter) haplosporosomes were present. A membrane bound body was often observed within the haplosporosomes (Figure 7.5). In the apical zone of the endosporoplasm a

spherule approximately 700 nm in diameter was formed by several vesicles (Figure 7.5). Dense vesicles (DVs) (Diameter = 159 nm to 228 nm (mean = 200 nm)) were numerous throughout the episporoplasm (Figure 7.5). Bundles of microfilaments were also occasionally observed in the episporoplasm as were lipid droplets. A lipid body was often observed adjacent to the middle of the spore (Figure 7.5). The wall of mature spores possessed neither ornamentation nor any attached cytoskeletal structures.

7.4 Discussion

The parasite described in this study shows the usual spore morphology, structure and ornamentation of a haplosporidian species. The internal organisation of the spore endosporoplasm is similar to other haplosporidian species. This was characterised by the presence of an equatorial nucleus, several electron dense membrane bound haplosporosomes and an apical spherule. The existence of an orifice that was covered with an operculum indicates the parasite belongs to *Minchinia, Haplosporidium* or *Bonamia*.

The parasite's identity was confirmed as the same parasite species characterised in Chapter 3 with an *in-situ* hybridisation assay utilising an oligonucleotide ISH probe (SSRDb) on an oyster from the same batch the *Minchinia occulta* n.sp spores were obtained. The assay did not react with any of the other haplosporidian parasites (*Minchinia teredinis*, *H. costale and H. nelsoni*). Individual parasites were also observed within the reproductive tissue of the host and were from the same host species and within the same range as the parasite described in Chapter 3.

The only previous mention of spore ornamentation in *M. occulta* n. sp was by Hine and Thorne (2002) in a TEM ultrastructural study of spores. Hine and Thorne (2002) noted the presence of surface microtubules present on the wall of mature spores. The spores observed in the current study, utilising both SEM and TEM, suggested these microtubules formed a covering of branching microtubules. The size and arrangement of the internal organisation of the spore observed here was consistent with those described previously by Hine and Thorne (2002).

The ornamentation on the spores of the rock oyster parasite was unique among described haplosporidian species. The spores of the rock oyster parasite do not have spore wall derived ornamentation and is therefore different from most described *Haplosporidium* species.

The branching network of microtubules on the spores of *M. occulta* resembles, at least superficially, the spore ornamentation of *Haplosporidium nelsoni* and *H. costale* in *Crassostrea virginica* (Burreson, E.M. and Reece, K.S., 2006). These species have ornamentation consisting of a network of branching fibres. However, the composition of the ornamentation differs considerably from the parasite described here. Spore ornamentation in *H. nelsoni* consists of fibres that are derived from the epispore cytoplasm and are tubular but with considerable internal complexity (Burreson, E.M. and Reece, K.S., 2006). In addition, the covering on *H. nelsoni* consists of individual ribbons tightly bound together that separate at the aboral end of the spore and do not cover the opercula lid unlike *M. occulta* n. sp (Burreson, E.M. and Reece, K.S., 2006). The fibres from *H. costale* while derived from the epispore cytoplasm, are not tubular and have a fine braided structure unlike *M. occulta* (Burreson, E.M. and Reece, K.S., 2006; Perkins, F.O., 1969). Ornamentation on spores of *Haplosporidium louisiana* consist of very thin filaments composed of spore wall material that wrap around the spore (Perkins, F.O., 1969). No spore wall derived ornamentation was observed on the spores of *M. occulta* n. sp.

Hine and Thorne (2002) suggested the microtubule-like structures derived from the episporoplasm in *M. occulta* appeared similar to microtubules in the epispore cytoplasmic vacuoles of *Minchinia* in crabs (Hine, P.M. and Thorne, T., 2002; Perkins,

F.O., 1975) and similar structures may be seen aligned under the epispore plasma membrane in *M. chitonis* (Ball, S.J., 1980; Hine, P.M. and Thorne, T., 2002). Bundles of similar microtubule-like structures also enter the ECE of *M. teredinis* (Hine, P.M. and Thorne, T., 2002; McGovern, E.R. and Burreson, E.M., 1990). Comps and Tige (1997) also reported conspicuous microtubule-like fibrils of 20 nm in diameter in the cytoplasmic tails of *Minchinia* sp. infecting the mussel *Mytilus galloprovincialis* on the Mediterranean coast of France. The microtubule-like structures were also visible in the episporoplasm surrounding the spore wall similar to *M. occulta* n. sp. except the structures were focally distributed and were aligned (Comps, M. and Tige, G., 1997). Similar unaligned microtubules have also been reported from *Haplosporidium ascidiarum* and *H. ascidiarum* also appears to lack spore wall filaments (Hine, P.M. and Thorne, T., 2002; Ormieres, R. and de Puytorac, P., 1968). Therefore, *H. ascidiarum* may also be a species of *Minchinia.*

However, *Minchinia occulta* n. sp. differs from other *Minchinia* species since *M. tapetis* infecting *Ruditapes decussatus* in Europe has a single posterior epispore cytoplasmic extension (ECE; Azevedo (2001)) while *M. teredinis* infecting shipworms (*Teredo* sp.) in the west Atlantic has four ECEs (McGovern, E.R. and Burreson, E.M., 1990). *Minchinia chitonis*, infecting the chiton *Lepidochitona cinereus* has two opposing ECEs (Ball, S.J., 1980). While the ornamentation of each of these *Minchinia* species is composed of epispore cytoplasm, the parasite observed here differs from each of these parasites since it does not appear to possess an ECE(s). Instead, it is entirely covered in branching microtubule-like structures derived from the epispore cytoplasm and possesses a ridge on one side of the spore (Figure 7.4). The ridge appears to be composed of a similar material as the microtubules and therefore may be a similar

ephemeral structure. No evidence of any epispore cytoplasmic extensions (ECEs) or tails were observed on spores of *Minchinia occulta* n. sp.

The results presented here suggest the rock oyster parasite should be assigned to *Minchinia* and not *Haplosporidium* or *Bonamia*. The parasite possesses microtubule-like structures similar to other *Minchinia* and there was a lack of any spore wall derived ornamentation that would suggest the parasite should be assigned to *Haplosporidium* or *Bonamia*. This is consistent with the results described from Chapter 3 where parasite fell within *Minchinia* in a phylogenetic analysis of the parasite's SSU rRNA gene and is a sister taxon to a clade composed of *M. chitonis*, *M. teredinis* and *Minchinia* sp. of *Cyrenoida floridana*.

Hine and Thorne (2002) described the development of *Minchinia occulta* n. sp. including multinucleate and spore forming stages. However, it is possible multi-nucleate stages are relatively rare in the life cycle of *Minchinia occulta* n. sp. At least four subsequent trips to the sites of the original infections failed to detect the parasite. It is likely *Minchinia occulta* n. sp. was present at the sites but as a single nucleate stage not easily recognised in histology. The samples obtained by Hine and Thorne (2002) were unusual for the parasite since they were obtained when mortalities were occurring and because subsequent sampling trips failed to detect the parasite (when no mortalities were occurring). Carnegie *et al* 2006 also described spore forming multi nucleate stages in *Bonamia perspora*. It may be the single nucleate stage of the life cycle becomes more dominant in the life cycle of the Haplosporidia the more closely related species become to the *Bonamia*.

No spore wall derived ornamentation was observed on the spores from the rock oyster parasite unlike the posterior thickening and spore wall derived filaments observed on *Haplosporidium hinei*. Consequently, the results presented in this Chapter confirm the pearl oyster and rock oyster haplosporidians are separate species and therefore the SSR69 probe must cross react to the pearl oyster parasite. The SSR69 probe underwent an assessment of its specificity with six other described haplosporidian species however, it is impossible to rule a cross reaction with an undescribed parasite species such as the pearl oyster parasite. These results suggest there is some sequence similarity between the two haplosporidians at the SSU rRNA gene site targeted by the SSR69 probe. The sequences obtained from two other separate regions of the SSU rRNA gene and the results obtained from the SSRDb ISH assay (Chapter 5) combined with the comparison of spore ornamentation presented in Chapters 6 and 7 suggest at least some of the *Haplosporidium hinei* pearl oyster infections were co-infections with *Minchinia occulta* n sp.

The definition of *Haplosporidium* has been confounded by a lack of knowledge of the spore ornamentation of the type species *H. scolopi* Caullery and Mesnil 1899. Characterisation of spore ornamentation requires both TEM and SEM examination. This has not been achieved for many haplosporidian species and it will probably require DNA sequence analysis of a large number of species in the phylum to elucidate the relationships among the different morphological types. The TEM and SEM analysis presented here confirms the molecular characterisation of Chapter 3 of a *Minchinia* parasite infecting rock oysters (*Saccostrea cucullata*) on the same coastline. Because deparaffinised tissue was used for SEM, the presence of epispore cytoplasm-derived projections typical of other *Minchinia* spp. cannot be ruled out.

Further analysis needs to be conducted with samples of infected oyster tissue specifically fixed for electron microscopy.

Chapter 8 : **The search for intermediate and alternative hosts.**

This chapter describes the preliminary attempts to identify intermediate and alternative hosts of the haplosporidian parasites described in the study. It also suggests further refinements in methods that may aid these objectives and provide fresh samples of the parasites

8.1 Introduction

Cascade Bay in King Sound in northern Australia last recorded a *Haplosporidium hinei* infection in 1995 while Willie Creek recorded an outbreak in 2000. In both of these cases, pearl oysters were never again deployed to the sites of the original infection. The site of the third infection, the Carnarvon pearl oyster hatchery, implemented management measures to prevent further infections. These measures appear to have been successful since no further infections were recorded. In all three outbreaks the entire batch of infected pearl oysters were culled. As a result, little is known about the pathogencity of the pearl oyster parasite. In addition, the sporadic nature of the outbreaks has limited research into the epidemiology of the infection but this feature alone indicates that an alternative host may be more important as a reservoir for maintaining the parasite.

An intermediate host is suspected in the life cycle of haplosporidian parasites since direct transmission has not been demonstrated in any *Minchinia* or *Haplosporidium* spp. parasite (Burreson, E.M. and Ford, S.E., 2004) . The widespread distribution of the pearl oyster outbreaks suggest that if an intermediate host(s) is required then it too is widely distributed.

The results obtained in Chapter 5 suggested a haplosporidian parasite (*Minchinia occulta*) detected in rock oysters may be co-infecting *Haplosporidium hinei* infected pearl oysters. A method of directly transmitting the parasite between rock oysters and juvenile pearl oysters would not only confirm the parasite is capable of infecting juvenile pearl oysters but would also allow a closer assessment of the threat the parasite poses to the pearling industry, and allow changes in the morphology of the parasite between hosts to be examined. By being able to demonstrate transmission of

the rock oyster parasite then the sample technique may be applied to attempt infection of pearl oysters with *Haplosporidium hinei* once a new infection is detected. In this way *Minchinia occulta* may be used as a model for the transmission and assessment of *Haplosporidium hinei*.

The development of sensitive molecular tools for the detection of haplosporidian parasites has a number of practical uses. These include attempting to determine the geographic range of the parasite, whether the parasite can be detected in alternative hosts and its mode of transmission from infected to uninfected oysters.

This study attempts the experimental transmission of *Minchinia occulta* from infected rock oysters to uninfected adult rock oysters and pearl oyster spat. The oysters were also co-housed with or without potential intermediate hosts. Pearl oysters were also bathed in homogenised digestive gland from infected rock oysters.

This chapter also describes the deployment of spat to a site where *Haplosporidium hinei* has been detected in the past; Cascade Bay (-16.59'S and 123.54'E). Detection of *Haplosporidium hinei* in this trial would allow monitoring of the clinical effects of the parasite on the oyster and provide fresh samples for sequencing of parasite DNA. Attempts were also made to locate the *Minchinia* and *Haplosporidium* parasite(s) from rock oyster and tropical oysters obtained from the sites where pearl oysters became infected with *Haplosporidium hinei*.

8.2 Materials and Methods

8.2.1 Infection trials

In this study, numerous trials were performed on the transmission of *Minchinia occulta* from rock oysters to rock oysters or pearl oyster spat. Possible horizontal transmission was tested firstly, by cohabitation secondly, with direct injection of infected tissues and thirdly, by cohabitation with a variety of invertebrate fauna from Cascade Bay and Northam in Western Australia. Live infected rock oysters were obtained from the Montebello Islands as in Chapter two. Uninfected pearl oyster spat (5 – 10mm) were obtained from the pearl oyster hatchery located in Broome, Western Australia. Hatchery spat were confirmed uninfected by PCR (SSF66/SSR69), ISH and histology. A variety of live invertebrate zooplankton species including copepods were obtained from a saline pond in Northam near Perth and Cascade Bay using a standard plankton net with a 20 µm mesh size. These samples were tested for the parasite by PCR and ISH using the methodology outlined in Chapters 3 and 4.

8.2.2 The inoculation experiments:

Oysters were maintained and fed in closed aquaria (40 L) containing full-strength salt water (35 g / L; Figure 8.1). The water temperature of the tanks was maintained at 23 °C. A sub-sample of rock oysters was used to confirm infection before the trial. Infection was confirmed using PCR with the primers SSF66/SSR69 and *in situ* hybridisation with the SSR69 and SSRDb oligonucleotide probes.

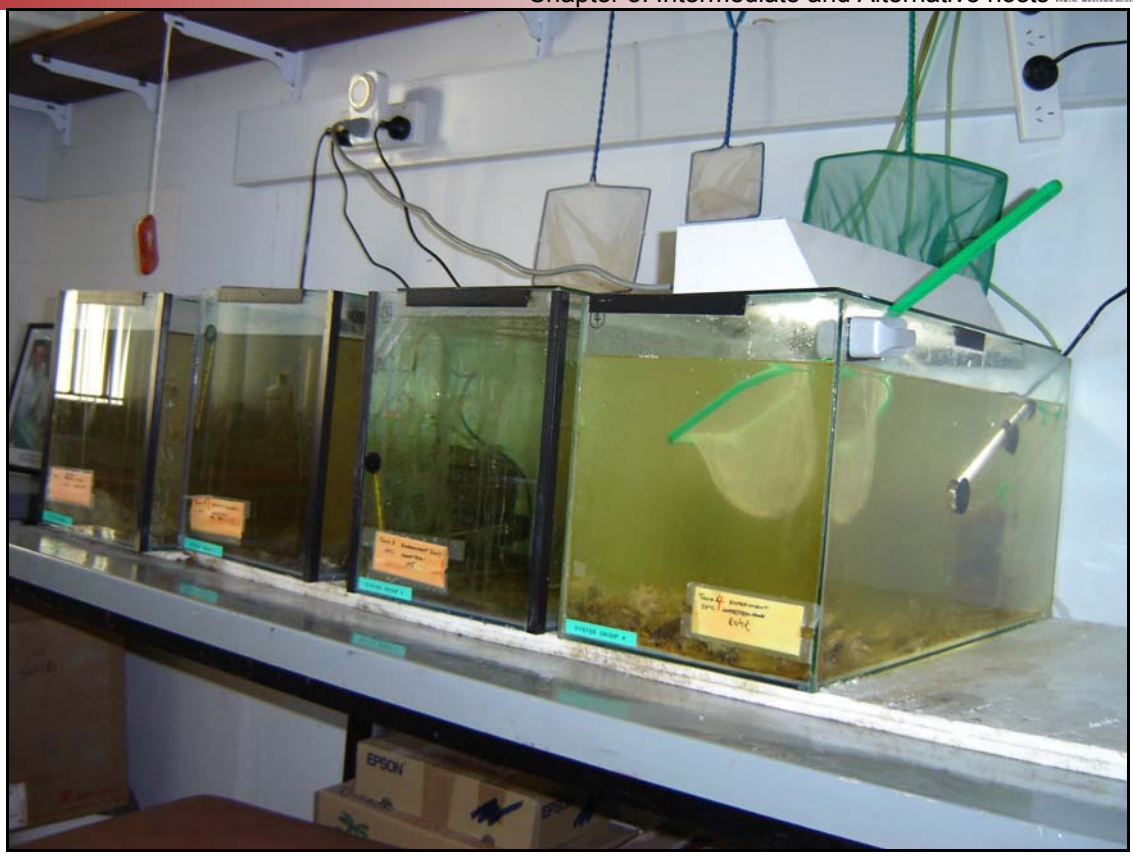


Figure 8.1 The 40 L aquaria used during the cross infection trial.

During the experiments, oysters were fed daily on 1 L of micro-algae mixture of the genera *Chaetocero*, *Isochrysis* and *Pavlova*. Starter cultures were obtained from Challenger TAFE College (Fremantle, Western Australia) and were maintained in 4 L glass bottles using F2 media and gro lux ^{tm} fluorescent tubes (Figure 8.2). Glass bottles were covered in foil to prevent contamination. An air tube was inserted into each culture to allow circulation. A second tube was inserted to allow air to escape. A 4 ml syringe was inserted to this tube and stuffed with cotton wool to prevent contamination (Figure 8.2). Culture media was autoclaved at 120°C at 20 atmosheres (ata) for one hour before use. Algae cultures were lit for 12 h per day.

Figure 8.2 The algal cultures used in the project. Three different species (*Chaetocero*, *Isochrysis* and *Pavlova)* were used to maintain live pearl and rock oysters at Murdoch University.

Oyster mortality was checked daily. Reproductive tissue and digestive glands from 15 rock oysters were homogenized in sterile salt water (35 g/ L) and used to inoculate the pearl oysters. For each trial, 30 oysters of the same origin were held as a negative control. Infection was assessed by histological examination and *in-situ* hybridisation. The experiments to artificially reproduce infection are listed in Table 8.1.

Trial 1

Thirty *Minchinia occulta* infected oysters were cohabitated with 120 pearl oysters (5 mm shell width) for three weeks in a 40 L aquarium. In a second aquarium, 30 infected rock oysters were cohabitated with 30 uninfected rock oysters from Broome in Western Australia (Table 8.1).

Trial 2

Minchinia occulta infected tissues were inoculated by forced injection with 0.05 ml of infected digestive gland tissue through the byssal notch of uninfected pearl oyster spat. A total of 120 pearl oyster spat 5 mm to 10 mm in shell width were injected (Table 8.1).

Trials 3 to 5

Transfer of *Minchinia occulta* from infected rock oysters was also attempted using an assortment of invertebrates from various sources. In these trials each aquarium was stocked with live infected oysters or faeces (pseudo and true) from infected oysters, a variety of plankton species including copepods and uninfected rock or pearl oysters (Table 8.1).

For the most part, the experimental conditions described above for the inoculation experiments were the same for the cohabitation experiments. The trials performed to reproduce the infection by cohabitation in experimental conditions are listed in Table 8.2.

Table 8.1 Experiments performed to transmit *Minchinia occulta* by cohabitation of infected rock oysters, uninfected rock oysters and uninfected pearl oysters.

Trial	n of infected rock oysters	Target Species (non infected)	Mode of transmission	n	Duration
1	30	Pearl oyster spat 5-10mm	Direct- cohabitation	100	12 weeks
1	30	Adult rock oysters	Direct- cohabitation	30	12 weeks
1	nil	Pearl oysters and rock oysters	None-negative control	60 and 30	12 weeks
2*	nil	Pearl oyster spat 5 mm	Injection of infected DG	120	3 weeks
2	nil	Pearl oyster spat 5mm	None- negative control	120	3 weeks
3	30	Pearl oyster spat 5 mm (and zooplankton)	Cohabitation with zooplankton (Saline pond-Northam)	60	12 weeks
3	30	Adult rock oysters	Cohabitation with zooplankton (Saline pond- Northam)	30	12 weeks
3	30	Adult rock oysters and pearl oyster spat	None- negative control	30 and 60	12 weeks
4	Nil pseudo-faeces only	Zooplankton (Cascade Bay)	By cohabitation with rock oyster pseudo faeces.	60	12 weeks
4	nil	Zooplankton (Cascade Bay)	None-negative control	-	
5	30	Pearl oyster spat 5 mm (and zooplankton)	Cohabitation with zooplankton (Cascade Bay King Sound)	60	12 weeks
5	30	Adult rock oysters (and zooplankton)	Cohabitation with zooplankton (Cascade Bay King Sound)	60	12 weeks
5	30	Adult rock oysters and pearl oyster spat	None- negative control	30 and 60	12 weeks

8.2.3 Infection by deployment of spat to a previous infection site and the sampling of rock oysters from the previous infection sites.

Six thousand pearl oyster spat were deployed to Cascade Bay (see Chapter 2) by long line in a manner similar to those of the original infection. These oysters were

deployed on the 22/1/2006 and sampled approximately every six months up until 12/4/2007. Rock oysters and pearl oysters (where possible) from each of the other previous infection sites (Willie Creek and Carnarvon) as well as Koolan Island were sampled as outlined in Chapter 2. All of the bivalves collected in the study were subjected to histological examination and the molecular methods including PCR (HAP; (Renault, T., Stokes, N.A., Chollet, B., Cochennec, N., Berthe, F., Gerard, A. and Burreson, E.M., 2000), protozoan (Carnegie, R.B., Meyer, G.R., Blackbourn, J., Cochennec-Laureau, N., Berthe, F. and Bower, S.M., 2003) and SSR69/SSF66 primers) and *in-situ* hybridisation (polynucleotide, SSR69 and SSRDb probes). These assays were performed as described in Chapter 4.

Plankton samples were obtained from the Montebello Islands and Cascade Bay using a standard plankton net with a 20 µm mesh size. The samples were fixed in 10% seawater buffered formalin. An alternative sample from each location was also obtained and stored in 100% ethanol.

8.2.4 Diagnosis of Minchinia occulta and Haplosporidium hinei.

Diagnosis of a haplosporidian infection for the aquaria infection trails, Cascade Bay spat deployment and the samples obtained from the previous infection sites was performed using techniques with varying levels of specificity so as to maximize the chances of obtaining positive results. The techniques used were histology, PCR (SSR69/SSF66 primer) and *in-situ* hybridisation (with the SSR69 and SSRDb probe) since these tests had already been shown to detect *Minchinia occulta* in rock oysters (Chapter 4). Each of these techniques was performed using the methods outlined in Chapters 3 and 4.

8.3 Results

8.3.1 Aquaria Infection Trials

Minchinia occulta transmission was not detected in either the cohabitation or forced injection experiments (Trials 1 and 2) by *in situ* hybridisation or histology (Table 8.3). Since these trials failed to produce an infection it was hypothesised that an intermediate host may be responsible for transmitting the parasite. Consequently, a number of cohabitation experiments were performed using plankton samples from various sources in order to attempt to find the intermediate host.

Table 8.2 Results of trials of experimental transmission of *Minchinia occulta* from infected rock oysters.

Trial	Source/route of infection	Target species	Result +/-*	n
1	Direct	Pearl oyster spat	-	60
1	Direct	Adult rock oysters	-	30
1	Direct		-	
2	Injection	Pearl oyster spat	-	100
2	n/a	Pearl oyster spat	-	100
			-	
3	Zooplankton (Northam)	Pearl oyster spat	-	45
3	Zooplankton (Northam)	Adult Rock oysters	-	30
3	n/a	Pearl oysters and rock oysters	-	30 and 30
			-	
4	Pseudo-faeces	Zooplankton (Cascade Bay)	-	2
4	n/a	Zooplankton (Cascade Bay)	-	2
			-	
5	Zooplankton (Cascade Bay)	Pearl oyster spat	-	60
5	Zooplankton (Cascade Bay)	Adult Rock oysters	-	30
5	n/a	Pearl and Rock oysters	-	30 and 60

* Infection status assessed by Histology, PCR (SSR69 and SSF66 primers) and *in – situ* hybridisation with the SSR69 labeled probe.

8.3.1 Infection by cohabitation

The experiment was successful in co-housing pearl oysters and rock oysters in closed aquaria for the duration of 12 weeks. Direct transmission of the parasite by cohabitation (without plankton) failed to produce an infection (Table 8.3). The forced injection of homogenized infected digestive gland into pearl oysters also did not produce an infection. As a result, live plankton samples containing a variety of invertebrate fauna including copepods were placed in the aquaria with the infected rock oysters or their faeces (true or pseudo). The zooplankton and oyster samples were then assessed for the parasite by PCR and ISH. In each of the different experiments transmission of the parasite failed to be detected (Table 8.3).

8.3.2 Pearl oyster samples from Cascade Bay and Willie Creek.

Adult wild pearl oysters from Willie Creek and spat from Cascade Bay produced negative results by histological examination, PCR and the oligonucleotide ISH (Table 8.4). PCR assays utilised the SSF66/SSR69, all HAP primers (Renault, T., Stokes, N.A., Chollet, B., Cochennec, N., Berthe, F., Gerard, A. and Burreson, E.M., 2000) and protozoan primers (Carnegie, R.B., Meyer, G.R., Blackbourn, J., Cochennec-Laureau, N., Berthe, F. and Bower, S.M., 2003). The spat did produce positive results by the non-specific polynucleotide ISH (Figure 8.3). The unidentified parasite was widely distributed through-out the digestive gland of the host and was present in each of the sampled batches at a prevalence of approximately 60% (Table 8.4). Of the seventy one oysters processed from the first sample forty one were positive (57 %) after only 6 six weeks deployment.

Table 8.3 Results of assays for pearl oyster samples from Cascade Bay and Willie Creek.

		Diagnostic Method			
Location	**Date**	**PCR***	**ISH – polynucleotide probe**	**ISH-oligonucleotide probe**	**Histology**
Cascade Bay#	14/3/06	0/45	41/71	0/71	0/171
	30/5/06	0/45	21/30	0/50	0/120
	22/9/06	0/45	23/34	0/50	0/124
	15/4/07	0/45	24/36	0/50	0/122
Willie Creek	8/5/06	0/20	11/20	0/20	0/20

* PCR with protozoan, HAP, and SSF66/SSR69 primers. # ISH with polynucleotide and oligonucleotide (SSR69) probes. # Longline was deployed 22/1/2006

The non-specific ISH probe used to target the parasite produced strong hybridisation signals with little background staining (Figure 8.3). This signal was not reproduced in tissues processed from uninfected oysters or in negative control material.

The unidentified parasites location in tissue sections were confirmed in H&E sections cut adjacent to those tested with *in situ* hybridisation (Figure 8.3). The parasites were primarily located in the connective tissue (Leydig) and digestive diverticula of the digestive gland with some individuals located in mantle connective tissue (Figure 8.3).

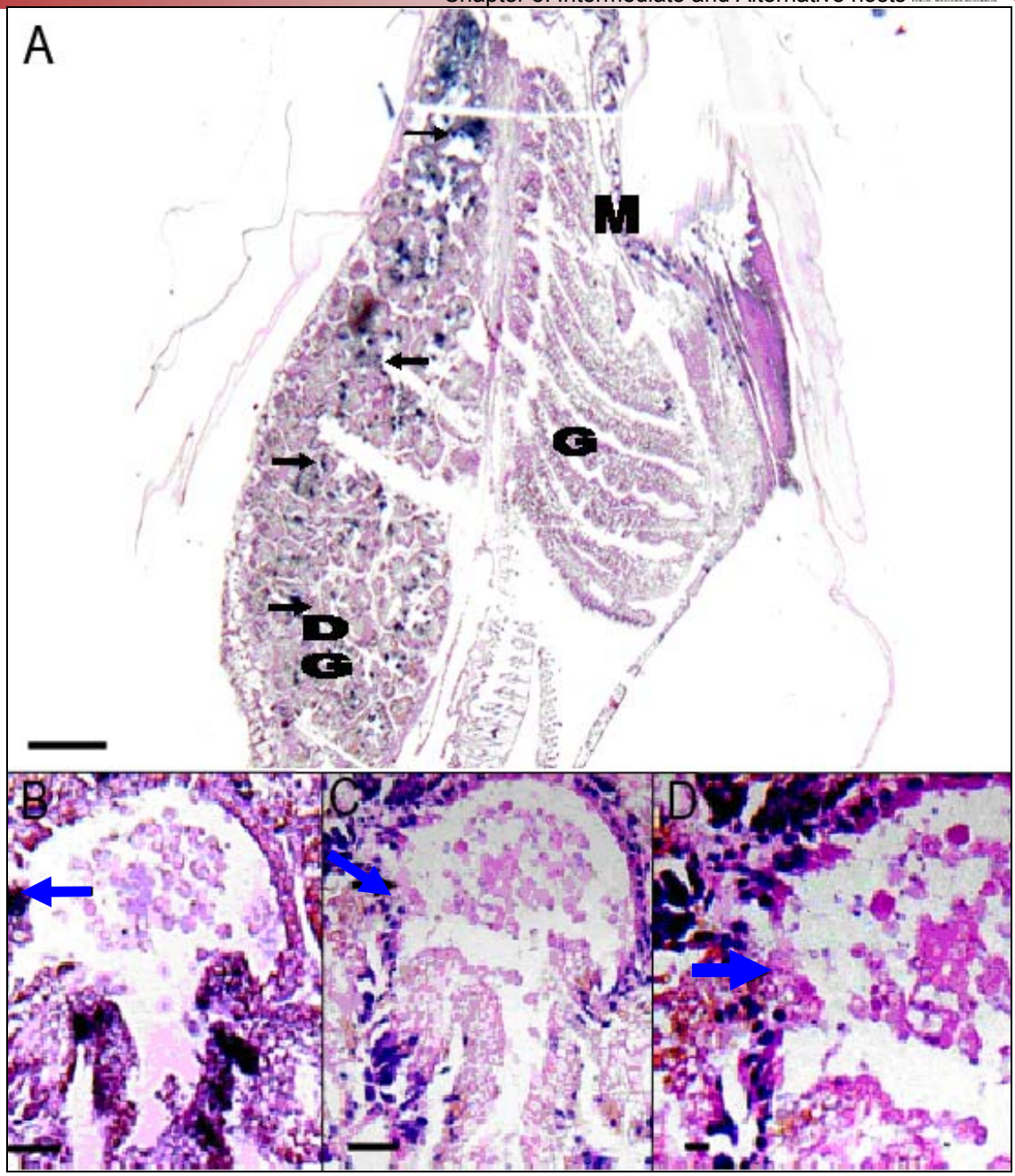


Figure 8.3 A section from a pearl oyster spat from Cascade Bay, King Sound assayed with a non-specific polynucleotide ISH. Digestive gland is indicated by a DG. The gills are indicated with a G while mantle tissue is indicated with a M. Parasites are indicated by a darker colouration. Sections are counterstained in a brazilian hematoxylin. Scale bar = 50 µm. (B) Higher magnification view of the hybridisation of the polynucleotide probe to the Cascade Bay parasite in tissue sections of the digestive gland. Not all parasites are present in the neighbouring section. Scale bar = 10 µm. (C) Adjacent section stained in H&E. Scale bar = 15um. (D) Infected section enlarged to show what appears to be a bi-nucleate life stage. Scale bar= 8 um.

The only parasite stages observed appeared to be single nucleate or bi nucleate stages (Figure 8.3). Oyster granulocytes were common among the unidentified parasites but phagocytosis or necrotic debris was not observed. Overall, the infected pearl oyster spat were growing strongly and appeared healthy with fully extended mantles (unpublished observations).

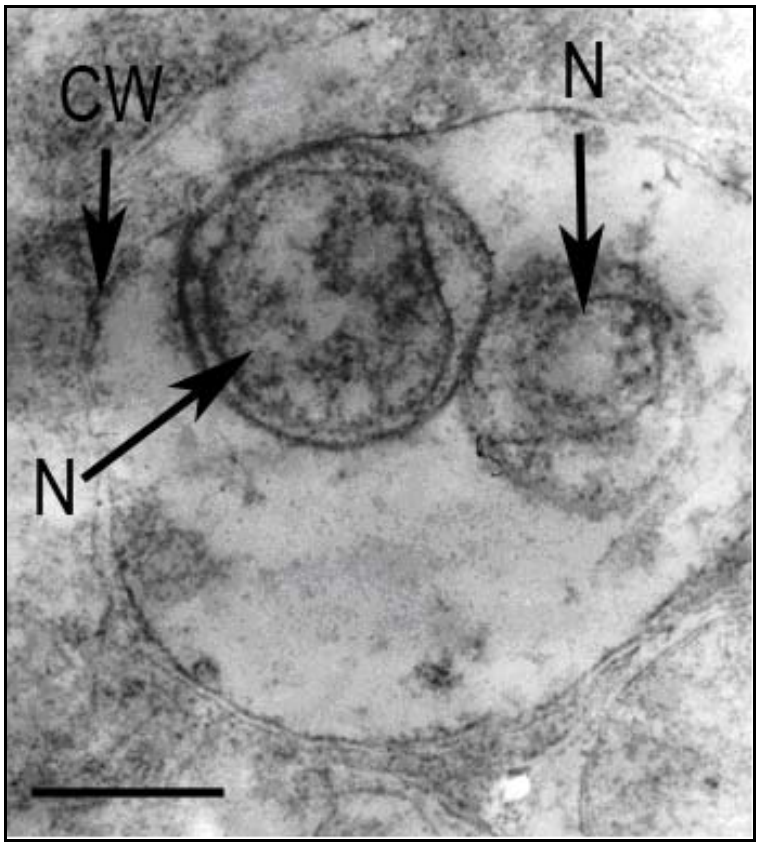


Figure 8.4 Transmission electron microscopy of the parasite detected in Pearl Oyster samples from Cascade Bay. CW denotes cell wall. N denotes a nucleus. Scale bar = 750 nm.

In order to attempt to identify these organisms TEM was performed. The parasite measured 3 µm in diameter and appeared to be a "microcell" parasite (Figure 8.4). However, no further diagnostic features could be obtained.

8.3.3 Rock oyster samples

The rock oyster samples from each of previous locations of the infection were tested by histological examination, PCR and ISH. None of the rock oyster samples from the previous infection sites tested positive (Table 8.4).

Table 8.4 Rock oyster (*Saccostrea cucullata*) and tropical oysters (*Saccostrea echinata*) sampled from the previous infection sites of *Haplosporidium hinei* in pearl oysters and Koolan Island.

Location	Species	Date collected	PCR*	ISH– polynucleotide probe	ISH- oligonucleotide probes (SSR69/SSRDb)	Histology
Cascade Bay	Rock oysters	14/3/06 and 30/5/06	0/25	0/72	0/72	0/72
Quondong Point (nr Willie Creek)	Rock oysters	10/5/06	0/25	0/65	0/65	0/65
Carnarvon	Rock oysters	1994	0/25	0/36	0/36	0/36
Koolan Island	Tropical oysters	1994	N/A	4/27	4/27	4/27

* With protozoan, HAP, and SSF66/SSR69 primers.

8.3.4 Koolan Island Tropical Oyster sample

Four of the 27 tropical oysters from the Koolan Island sample tested positive with both of the specific oligonucleotide ISH probes. Histological examination indicated a multinucleated haplosporidian parasite was present in the samples (Figure 8.5).

The parasite was identified in hematoxylin-eosin stained sections using *in-situ* hybridisation with the SSR69 oligonucleotide probe. The probe produced strong

hybridisation signals with little background staining (Figure 8.5). This signal was not reproduced in tissues processed from uninfected oysters or in negative control material (Figure 8.5)

The parasite measured 4 µm in diameter and was disseminated in the mantle and gill tissue of the host. The only parasite life stages observed were multinucleated plasmodia.

There appeared to be little in the way of a defensive response from the oyster to the parasite. Indeed, there appeared to be few haemocytes in the vicinity of the parasites. No phagocytosis of parasite cells was observed. Spores were not detected in this study so the parasite was not able to be identified using the established morphological criteria (Burreson, E.M., 2001) and only formalin fixed material was available making molecular analysis difficult.

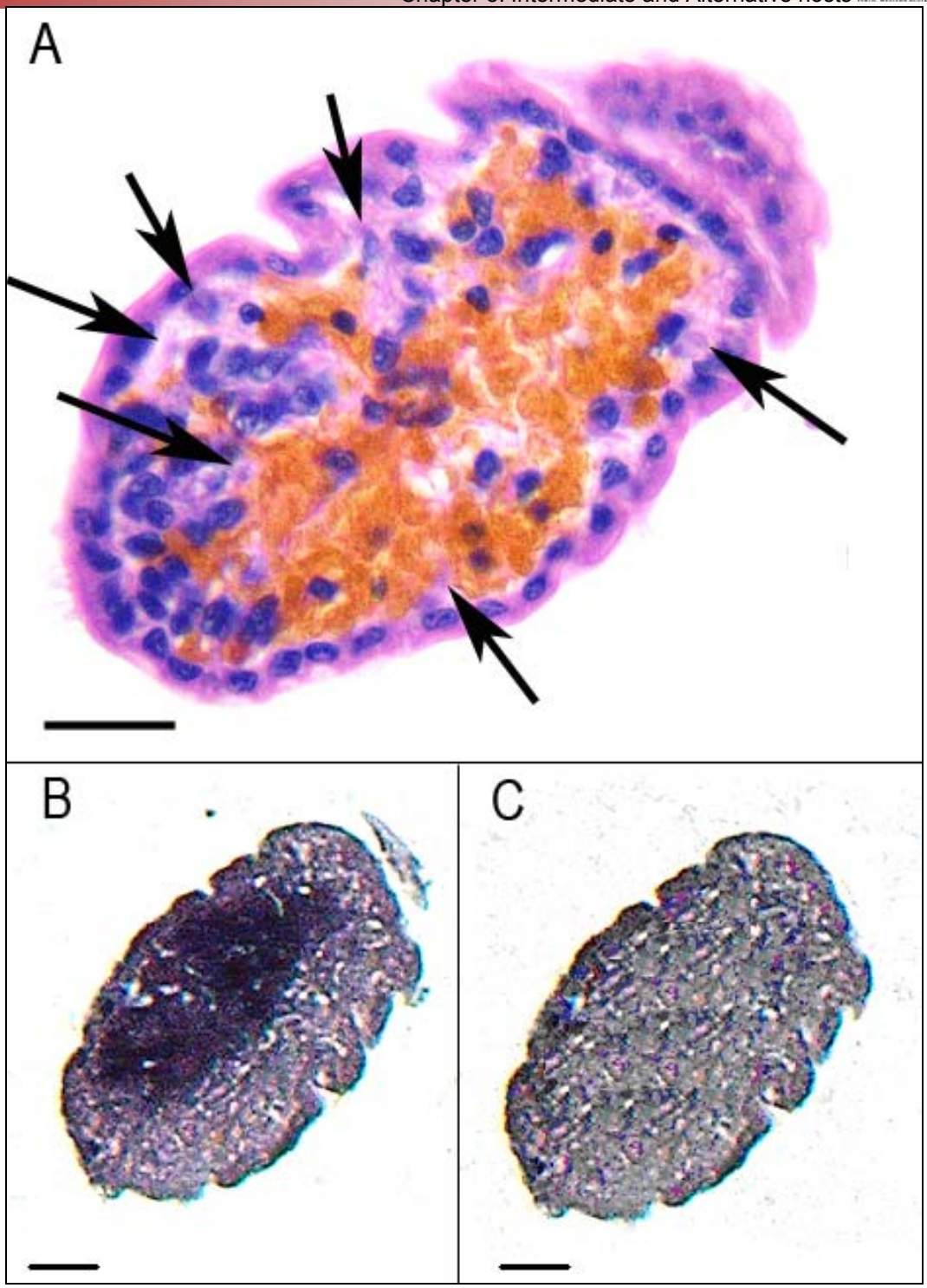


Figure 8.5 Serial sections of tropical oyster mantle tissue containing a haplosporidian parasite. (A) a hematoxylin-eosin stained section. Parasites are indicated with an arrow. (B) an *in situ* hybridisation assay containing the SSRDb probe. (C) an *in situ* hybridisation assay with no probe in the hybridisation solution (negative control). Sections are counterstained in a brazilian hematoxylin. Scale bars = 10 µm.

8.4 Discussion

8.4.1 The inoculation experiments

Transmission of *Minchinia occulta* could not be achieved by the methods used in this study. Since no transmission occurred it is probable *Minchinia occulta* uses an intermediate host in its lifecycle. This finding is similar to findings for haplosporidian parasites overseas (Burreson, E.M. and Ford, S.E., 2004).

Transmission of the haplosporidian *Bonamia ostrea* was recently performed by cohabitation with the brittle star, *Ophiothrix fragilis* (Lynch, S.A. *et al.*, 2007). *Bonamia ostreae*, in which spores have not been detected, has been shown to be directly transmissible by cohabitation with infected oysters (Elston, R.A., Farley, C.A. and Kent, M.L., 1986) or by inoculation (Hervio, D. *et al.*, 1985). Direct transmission of other spore forming haplosporidian parasites has not yet been demonstrated leading to speculation of an intermediate host. Direct transmission of another "microcell" parasite *Marteilia refringens* has also been unsuccessful (Berthe, F. *et al.*, 1998). However, the parasite was detected in the copepod *Paracartia grani* (Audemard, C. *et al.*, 2001) suggesting this species acts as an intermediate host. It has also been suggested that the infective stage of *Haplosporidium nelsoni* is capable of passing through a 1000 µm filter (Sunila, I. *et al.*, 2000) and a 150 µm filter (Ford, S.E. *et al.*, 2001) but not a 1 µm filter with UV irradiation. The widespread distribution of *Haplosporidium nelsoni* suggests either the parasite can infect a variety of intermediate hosts and/or the intermediate host is also widely distributed. It is also possible the intermediate host of *Minchinia occulta* is a similar resident(s) of the zooplankton such as a copepod and is widely distributed on the Western Australian coastline or more than one intermediate host exists for the parasite.

The plankton samples obtained during this study tested negative to the parasite by PCR and ISH.

Transmission under laboratory conditions could fail because either the environment or the oysters are not providing the right stimuli. The local environment may play a role in the virulence of the pathogen (Berthe, F., Pernas, M., Zerabib, M., Haffner, P., Thebault, A. and Figueras, A.J., 1998). It is possible that *Minchinia occulta* is a very fastidious parasite that requires very specific conditions to infect rock oysters or pearl oysters. These conditions may not have been met in the course of these experiments. It is also possible the spores may need a period of maturation in the environment although the trials including oyster faeces also failed to transmit the parasite. Further surveys of wild stocks of rock oysters and other bivalves would be desirable to examine the natural distribution of the parasite and examine possible sources of infection.

Direct injection of the parasite should have overcome many of the environmental variables or stimuli that may have prevented transmission. The failure of the direct injection experiments suggests some form of parasite maturation in an intermediate host is required. The preferred method to examine this hypothesis would be to identify the parasite stages of maturation and requirements directly in the intermediate host. Obviously, this would require identification of the intermediate host.

Identification of the intermediate host may be achieved by exposing hatchery raised oyster spat in aquaria to filtered sea water at the infection sites where the parasite is known to exist. The levels of filtration could be varied across a number of replicate aquaria. Once an infection was detected then an assessment of the potential

intermediate hosts that occur in the aquarium could be carried out. This would drastically reduce the number of potential intermediate hosts and the size of the intermediate host could be estimated. This method would be more effective than co-housing affected and unaffected oysters however; it relies on the intermediate host being present in reasonable numbers in the affected aquaria to facilitate its detection and identification.

The Cascade deployment of pearl oyster spat was the first time pearl oysters have been deployed to the site of a previous haplosporidian infection. The trial produced positive results with the non-specific polynucleotide probe. The same samples were negative with the more specific SSR69 and SSRDb oligonucleotide probes and PCR. Therefore, the parasite detected is unlikely to be *Haplosporidium hinei* or *Minchinia occulta* but another unidentified "microcell" parasite of pearl oysters. The prevalence of infection did not change during the trial and the parasite was also detected in adult pearl oysters from Willie Creek.

The distribution of the parasite in rock oysters does not match the locations of the parasite has been detected in pearl oysters. Thus far the parasite has not been detected in rock oysters from Willie Creek near Broome or at Cascade Bay in King Sound and further sampling may be required from these areas. It is possible the number of samples obtained from the sites was not adequate to detect a low prevalence of parasite (depending on the sensitivity of the tests employed; (OIE, 2006)). Alternatively, samples may need to be obtained at different times of the year. However, four of 27 tropical oysters were positive by the SSRDb oligonucleotide ISH probe. These oysters were obtained from Koolan Island north of King Sound. It

therefore appears likely that the original reservoir host of the *Minchinia occulta* infections in pearl oysters was tropical oysters. Further sampling and research is required to obtain ethanol stored infected tropical oysters for molecular analysis.

Chapter 9 General Discussion

The primary objectives of this study were to describe the haplosporidian parasites of northern Western Australia. Appropriate molecular, pathological and electron microscopy techniques were used. The specific aims were to use these methods to:

- Detect the haplosporidian parasite in rock oysters from north Western Australia.
- Suggest potential molecular assays for the rock oyster parasite and commence an assessment of their sensitivity and specificity.
- Determine whether the detected parasite was present in past haplosporidian outbreaks in pearl oysters using the PCR and ISH assays developed.
- Describe the spore ornamentation of the haplosporidian in pearl oysters using both scanning and transmission electron microscopy and compare the results to other haplosporidians and the molecular data obtained.
- Describe the spore ornamentation of the rock oyster haplosporidian using both transmission and scanning electron microscopy and compare the results to the pearl oyster haplosporidian, other haplosporidians and the molecular data obtained.
- Attempt to infect pearl oysters and rock oysters with *Minchinia occulta* by co-housing uninfected rock oysters and pearl oysters with infected rock oysters. Attempts were also made to obtain *Haplosporidium hinei* infected pearl oysters by deploying pearl oyster spat at a site were *Haplosporidium hinei* has been previously detected.

9.1 Detecting the haplosporidian parasite in rock oysters from north Western Australia

The results obtained here demonstrate through PCR and *in situ* hybridisation the presence of a cryptic haplosporidian parasite in rock oysters. The parasite was originally described as belonging to *Haplosporidium* however; a phylogenetic analysis indicated a *Minchinia* species between *Minchinia tapetis* and *Minchinia chitonis* (Figure 3.6). The parasites were generally present at low levels of infection making them difficult to detect by histological examination alone. The results of the PCR and *in-situ* hybridisation assays supported one another. These molecular techniques arrived at the same conclusion by independent means and therefore gave more confidence to these findings. Parasites were detected in the reproductive follicles, digestive gland and gills of the host. The *Minchinia* parasite detected in this study was at a relatively high rate of prevalence at 43% although parasite stages were restricted primarily to a single nucleate stage resembling a *Bonamia* species (Table 3.2).

The samples obtained in Hine and Thorne (2002) were unusual in that they were associated with mortalities (up to 80%) detected by energy companies operating on the north-west gas shelf of Western Australia. At least four subsequent attempts to detect the parasite failed to detect parasite suggesting the widespread mortalities described by energy companies were unusual. Oysters obtained in this study were subjected to considerably lower parasite loads than those described in Hine and Thorne (2002) and there was also a difference in the oyster tissues infected. In Hine and Thorne (2002) the parasite consisted of a variety of life stages including multi-nucleated plasmodia, sporoblasts and spores. It is possible the process of sporogenesis in *Minchinia occulta* is linked to the documented mortalities in rock oysters described by energy companies. Hine and Thorne (2002) found *Minchinia occulta* in a variety of anatomic locations

including the digestive gland, gills, mantle and to a much lesser extent the reproductive tissue of the host. In this study the parasite occupied primarily the reproductive tissues although some parasites were detected in the gills and digestive glands.

The current samples suggest the parasite is not causing mortalities in the infected oysters however; the parasite has been linked to mortalities of up to 80% in a previous study (Hine, P.M. and Thorne, T., 2002). The pathogenicity of the parasite in rock oysters and in the secondary host may be dependent on seasonal factors that are probably linked to sporogenesis in *Minchinia occulta*. It is not unusual for the pathogenicity of haplosporidian parasites to vary markedly according to external factors. *Bonamia roughleyi*, the aetiological agent of winter mortality in Sydney Rock Oysters within New South Wales, is known to cause mortality, rather predictably, in the winter months. The pathogenicity of *Haplosporidium nelsoni* (MSX) and *Perkinsus marinus* (dermo disease) in the Eastern oyster in the United States are known to intensify during periods of higher temperatures and salinity. Indeed, aquaculturists attempting to prevent infection or treat affected eastern oysters often translocate their oysters to an alternative site of lower salinity (Burreson, E.M. and Ford, S.E., 2004). It is possible sporogenesis in *Minchinia occulta* results in the mortality of the rock oyster. Consequently, the onset of mortalities in the rock oyster would be determined by the disease and factors that cause *Minchinia occulta* to sporulate. This hypothesis explains why Hine and Thorne (2002) found spores when the oysters were undergoing mortalities and why this study and other sampling trips that occurred when few mortalities where occurring did not detect *Minchinia occulta* spores.

This study did not attempt to determine pathogenicity. To do so would require assessing the link between the presence of the *Minchinia* to the occurrence of the disease as required in Evan's postulates or a similar scheme (Evans, 1976). This requires the presence of the disease and therefore any association cannot be assessed until disease is detected. It's likely year round sampling of the affected rock oysters may shed some light on its possible pathogenicity since these studies would better describe the life-cycle of the *Minchinia* and any associated mortalities. Once disease is detected comparisons can be made with the prevalence of the parasite and parasite load to the presence of disease. These studies should describe the spatial and temporal distribution of the disease as well as the parasite and could be combined with studies into any association between parasite load and the presence of disease. The results should determine if the parasite and disease association fulfills the requirements of Evan's postulates (Evans, 1976) and documents the pathogencity of the parasite. Alternatively, *in vitro* infection could be attempted in aquaria (Chapter 8). In these experiments the disease should occur more frequently in those exposed to the *Minchinia* with a spectrum of host responses along a logical biological gradient from mild to severe following exposure to the *Minchinia*.

9.2 Suggesting a potential molecular assay for the rock oyster parasite and commencing an assessment of their sensitivity and specificity.

Infection by *Minchinia occulta* in *S. cuccullata* was detected by histological examination (an initial examination and a retrospective examination) PCR and *in-situ* hybridisation with the oligonucleotide probe. The initial histological examination detected 14 infected oysters among the 56 oysters examined while PCR amplification detected 26 infected oysters (Table 4.3). *In-situ* hybridisation detected a total of 29

infected oysters (Table 4.3). These results indicate that both the PCR assay developed in this study and the *in-situ* hybridisation assay developed in Chapter 3 were capable of detecting *Minchinia occulta* in *S. cuccullata*. The comparison of diagnostic techniques suggested the ISH assay was the most sensitive followed by PCR (Tables 4.3 and 4.4). The oligonucleotide SSR69 ISH assay did not cross react with any of the six other haplosporidian species tested. However, results obtained in chapters 5, 6 and 7 suggest the SSR69 probe cross reacts with *H. hinei*. Consequently, a new probe was designed (SSRDb) for use in ISH assays.

The molecular tools described here provide a method to explore the pathogenicity of the parasite. The ISH assay could be used to pin point life cycle and pathological and anatomical relationships in either experimental (if successfully performed) or natural disease. The PCR assay developed in this study could be used to address questions about parasite presence while if a quantitative PCR such as real time PCR (rtPCR) was developed then this assay could be used to address questions of parasite load. Thus, these molecular tools could be used to address the requirements of parasite and disease association as in Evan's postulates (Evans, 1976).

It's possible the life cycle of the parasite is altered by seasonal factors such as the host life-cycle or external factors such as temperature. Presumably these factors could be used to "treat" affected oysters. The molecular tools described in this study may be used to assess the prevalence and load of the parasite in these attempted treatments. Any fall in the occurrence of the disease and the prevalence of the parasite would also provide evidence of the link between the parasite and associated mortalities and therefore help to fulfill Evan's postulates.

9.3 Determining whether the detected parasite is present in past haplosporidian outbreaks in pearl oysters.

The haplosporidian infection of pearl oysters tested positive by two different PCR assays for the *Minchinia* parasite with no evidence of contamination. The PCR assays suggest the infections were either a co-infection of two different parasites or that the pearl oyster and rock oyster parasites were the same species. The best way to resolve these differing hypotheses was to directly assess parasite spore morphology by both scanning and transmission electron microscopy (see 9.4 and 9.5 below).

In-situ hybridisation was employed to allow visualization of the parasites. Two oligonucleotide ISH assays were utilised; one utilised the SSR69 probe and reacted to all haplosporidian parasites in the infected pearl oysters while the other utilised the SSRDb probe and only reacted to some. Neither probe reacted to closely related haplosporidians *Haplosporidium nelsoni*, *Haplosporidium costale* or *Minchinia teredinis*.

Molecular methods such as *in situ* hybridization could be used to provide evidence for a co-infection or for same species identification. However, these methods were not as effective as electron microscopy in this study since samples suitable for molecular analysis of the pearl oyster parasite were not available or at least no sequence could be obtained from the samples that were available. This limited the sequence information that could be developed for the parasite making the interpretation of *in-situ* hybridisation assays difficult. If an *in-situ* hybridisation probe failed to react it was difficult to determine if the detected underlying sequence variation was the result of

intra or inter specific differences. The results of this study suggest there were four intra-specific polymorphic sites within the SSU rRNA gene sequence obtained in this study (Chapter 4). Alternatively, if the ISH probe annealed then it was difficult to rule out a chance cross reaction with an undescribed species since they may possess some sequence similarities at the site targeted. It may mean a more variable region such as the ITS region utilized for the detection and identification of *Perkinsus* and *Marteilia* species would need to be targeted (Audemard, C. *et al.*, 2004; Kleeman, S.N. and Adlard, R.D., 2000; Kleeman, S.N., Roux, F.L., Berthe, F. and Adlard, R.D., 2002) . In these circumstances species boundaries should be clearly defined before molecular analysis.

9.4 Describing the spore ornamentation of the haplosporidian in pearl oysters using both scanning and transmission electron microscopy.

The parasite described in this study showed the typical morphology, spore structure and spore ornamentation of a haplosporidian species. The internal organisation of the spore endosporoplasm shows a similar arrangement to other haplosporidian species. This was characterised by the presence of a basal or equatorial nucleus, an apical spherule and several electron dense membrane bound haplosporosomes. The presence of an orifice which is covered with an operculum suggests the parasite belongs to either *Minchinia* or *Haplosporidium (*or *Bonamia)*.

If the criteria proposed by Ormieres (1980) for distinguishing the genera *Haplosporidium* and *Minchinia* are followed then *Haplosporidium hinei* n.sp was correctly assigned to *Haplosporidium* by Hine and Thorne (1998) because the filaments are derived from the spore wall and not from epispore cytoplasm. The

ornamentation on the spores of the pearl oyster parasite described by the number of filaments, their length, insertion points on the wall and organisation of the filaments was unique among described haplosporidian species where spore ornamentation is known. The spores of the parasite have two spore wall filaments wound around the spore which appeared to originate from two posterior wall thickening. The parasite was named *Haplosporidium hinei*.

Molecular characterisation of the pearl oyster parasite will require samples suitable for molecular analysis. This would be best achieved using the replicate ethanol and formalin fixed sampling regime described in Chapter 2 the next time *Haplosporidium hinei* is detected. This type of sampling regime facilitates the collection of molecular data in samples where low levels of parasite prevalence have been detected. Molecular tools could then be developed and a phylogenetic analysis performed. In addition, the outbreak should be quarantined so that the pathogenicity of the parasite can be determined using the techniques discussed for the rock oyster parasite in section 9.1 above. In this way, the disease risk posed by *Haplosporidium hinei* can be properly assessed.

9.5 Describing the spore ornamentation of the haplosporidian in rock oysters using both scanning and transmission electron microscopy.

The parasite spores showed the typical morphology and structure of a haplosporidian species with the presence of an orifice that was covered with an operculum. The spore ornamentation consisted of a network of branching microtubule-like structures covering the entire spore including the opercula lid. A ridge was also present on one side of the spore beginning just below the operculum and ending approximately 2 um from the aboral end of the spore. No evidence of any spore wall derived

ornamentation could be detected. These results confirm the *Minchinia* sequence obtained from the rock oyster samples in Chapter 3 and confirm the pearl oyster and rock oyster parasites are different species.

Hine and Thorne (2002) described the development of *Minchinia occulta* including multinucleate and spore forming stages. However, it is possible multi-nucleate stages are relatively rare in the life-cycle of *Minchinia occulta*. At least four subsequent trips to the sites of the original infections failed to detect the parasite. It is likely *Minchinia occulta* was present during these subsequent sampling trips but the single nucleate stage was not easily recognised in histology. The single nucleate stage of the parasite was only recognised during this study by utilising *in-situ* hybridisation to explain positive PCR assays with degenerate HAP primers. Once described the single nucleate stage was easily detected in histology. The samples obtained by Hine and Thorne (2002) were unusual since they were obtained when mortalities were occurring and because subsequent sampling trips failed to detect the parasite (when no mortalities were occurring). Carnegie et al (2006) also described spore forming multi-nucleate stages in *Bonamia perspora*. Speculatively, the single nucleate stage of the life cycle may become more dominant in the life cycle of the haplosporidians the more closely related the parasite is to *Bonamia*. These results confirm phylogenetic analyses indicating *Minchinia* is a sister taxon to *Bonamia*.

9.6 Attempts to infect pearl oysters and rock oysters with the parasite by co-housing uninfected rock oysters and pearl oysters or with infected rock oysters and the search for the intermediate and reservoir host.

Transmission of *Minchinia occulta* in *S. cuccullata* could not be achieved by the methods used in this study. The infectivity trials indicated the parasite was not directly

transferable between pearl oysters and rock oysters or between rock oysters and rock oysters. These results indicate an intermediate host may be required to transfer the parasite. A survey of rock oysters from the sites where the pearl oyster parasite had been detected in the past did not find any evidence of either parasite. Also, the Cascade Bay deployment failed to produce a *Haplosporidium hinei* or *Minchinia occulta* infection and suggests the parasites were either not present at the site or the conditions required for the infection of pearl oysters were not met. The current lease holder of the Willie Creek site has now deployed spat to the site for 10 months. *Haplosporidium hinei* has not been detected to date. The lack of any detection of the parasite at Cascade Bay and Willie Creek suggests the original outbreaks of *Haplosporidium hinei* were unusual events. The widespread geographic distribution but sporadic frequency of the outbreaks confirms this finding.

The study did find what appeared to be a *Minchinia occulta* infection in tropical oysters further north at Koolan Island. The parasite was identified with both the SSRDb and SSR69 assays and diagnosis was confirmed on H/E stained sections (Figure 5.8). This result indicates the *M. occulta* was widespread on the Western Australian coastline although not necessarily in rock oysters (*S. cuccullata*). The parasite was not detected in plankton samples from Cascade Bay and the Montebello Islands by PCR or ISH.

The inability of obtain *Haplosporidium hinei* infected oysters from Cascade Bay limited studies into the pathogenicity of the parasite and restricted samples available for molecular analysis to formalin fixed archived samples. Attempts to transmit *Minchinia occulta* also failed and this hampered attempts to determine the pathogencity of this

parasite in rock and pearl oysters and to use it as a model for *Haplosporidium hinei*. It is probable only a small number of potential intermediate hosts were tested relative to the large number of species that may act as an intermediate hosts for haplosporidian parasites. This variety is the most likely reason no intermediate host has been detected for any of *Haplosporidium* or *Minchinia*. In addition, the factors that have limited *Haplosporidium hinei* outbreaks in pearl oysters to three detected outbreaks in pearl oysters despite its widespread distribution, would also limit the parasites transmission in infection trials.

One possible method that might produce an intermediate host would be to expose hatchery raised oyster spat in aquaria to filtered sea water at the infection sites where the parasite is known to exist. The grade of filtration could be varied across a number of replicates. Once an infection was detected then an assessment of the potential intermediate hosts that exist in the aquarium could be made in the aquarium where smallest grade of filtration was carried out. In this way the number of potential intermediate hosts would be drastically reduced and the size of the intermediate host could be estimated. This method would be more effective than co-housing affected and unaffected oysters. The presence of the *Minchinia* in the intermediate host could be assessed through the PCR and *in-situ* hybridisation assays developed in this study.

9.7 Overall

Evidence presented in Chapter 5 suggested the possibility the rock oyster and pearl oyster parasites were the same species. This evidence included sequences generated from multiple amplifications of the SSU rRNA gene with no evidence of contamination (Figure 5.2). An oligonucleotide ISH assay using the SSR69 probe also

confirmed these results (Figures 5.7 and 5.8). The SSR69 probe did not react to any of the six haplosporidian species tested. However, the assay utilising the SSRDb probe reacted to only some of the haplosporidian parasites present and did not react to the three haplosporidian species tested (Figures 4.7, 4.8 and 5.9).

The morphological evidence presented in Chapters 7 and 8 confirms the two parasites are different species. The principal evidence for this difference is the presence of a wrapping of branching microtubule like structures present on the surface of the spores and the lack of any spore wall derived ornamentation on the spores of *Minchinia occulta.*

Before this study, a single preliminary TEM description of the rock oyster and pearl oyster parasites had been carried out. Both parasites were described simply as *Haplosporidium* sp. It was not clear whether either parasite was one of those notifable under the OIE guidelines such as *H. nelsoni*. The results of this study confirm both parasite species are unique. The research presented here obtained samples of *Minchinia occulta* for both molecular and histological assessment. The SSU rRNA gene of the rock oyster parasite was sequenced allowing phylogenetic assessment and the development of molecular tools for its detection and description. The parasite was present at all four sampling locations at the Montebello Islands. *In-situ* hybridisation combined with histological analysis allowed the description of a cryptic uni-nucleate stage residing in the reproductive tissue of the host. The uni-nucleate stage resembles a *Bonamia* parasite which is a sister taxon to the *Minchinia*. The non-spore stages of the *Minchinia* remain under described in the literature perhaps because of the presence of cryptic uni-nucleate stages in their lifecycle. This study

has provided the tools for the easy detection of the *Minchinia* parasite of rock oysters in the future and described the spore morphology of both the pearl oyster and rock oyster parasites using TEM and SEM, in the process naming both species. As a consequence of this research, there are now several areas of future research into these parasites that need to be pursued. Formost amongst them is obtaining an SSU rRNA gene sequence for *Haplosporidium hinei*. The replicate sampling method outlined in Chapter 2 will facilitate this objective once the parasite is again detected. Future research should also attempt to determine the pathogenicity of both parasites using Evans postulates (Evans, 1976) or a similar scheme combined with year round sampling.

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Chapter 10 Appendix

10.1 Appendix 1: *Minchinia occulta* SSU rRNA sequence and position of the primers and probes used in the study.

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   1  aacacgtttg tactgttaaa ctgcagatgg ctcattaaaa cagttacagt ttatttgaca
  61  tttgaagtaa tgcagataac cgtggtaact tagggctaat acgtgacgaa aatcctgctt
 121 gcaggaggtg cttattagct taaaaccaat tgtggttgaa aaataataaa taacggatcg
 181 cgttggcttt gccggcgact tgtcattaaa atttctgact tatcagctag acggtaaggt
 241 attggcttac cgtggctatg acgagtaacg gggaattttg gttcgattcc ggagaggcag
 301 cctgagaaac ggctaccaca tccacggaag gcagcaggtg cgcaaattgc tcaatgttga
 361 ctaaacgaag cagtgaccag gcataacgag agcagctgca aagttgcgat ttggaatgag
 421 aacaatgtaa aagccttatc gattaacaag cggagggcaa gtctggtgcc agcagccgcg
 481 gtaataccag ctccgctagc gtatattaaa gttgttgctg ttaaaaagct cgtagttgga
 541 tatcagggcg ccgcgcgatg cccagccgta tcggctgcgc gcaaggctaa cgccgccgcg
 601 cggcgccctt acttcaggag catctatctg gccattgaat tggctgggtc tttggtcctg
 661 atcttttact ttgagaaaat taaagtgctc aaagcaggca gccgcctgga tgcattagca
 721 tggaataata agacgagact ttggcgccac gcatgtggac gttttgttgg ttttgggctg
 781 aagtaatggt tgataggaac aattgggggt gctagtatcg ccgggctaga ggtaaaattc
 841 tttgattccg gtgagactga cttatgcgaa agcattcacc aagtttgttt tctttaatca
 901 agaactaaag ttgggggatc gaagacgatc agataccgtc gtagtcccaa ctataaacca
 961 tgtcgactaa gtgttgggca agttttgctt gctcagaact ttgcgagaaa tcaaagtttt
1021 cggactcagg gggaagtatg ctcgcaagag tgaaacttaa aggaattgac ggatgggcac
1081 cacaagttgt ggagcctgcg gcttaatttg attcaacacg ggaaaccttc ccaagtccag
1141 acatagcaag gattgacaga ttcaagttct ttcttgattc tatgcatggt ggtgcatggc
1201 cgttcttagt tggtggagtg atctgtctgg ttaattccga taacgaacga gacctcagcc
1261 atctaaatag ctgtcgctaa cgcgttagcg cccgctcgcg ggctcagctt cttagaggga
1321 ccgtctgtgt ctagcagaag gaggtttgag gcaataacag gtcagtgatg cccttagatg
1381 ctctgggctg cacgcgcgct acaatgaggc acgcagcgag ttcctggctc gcgagagctg
1441 gggaatctgc aaagcgcctc aaagtgggga taggtgattg caattattca tcatgaacaa
1501 ggaatatcta gtaaacgcaa gtcatcaact tgcattgatt acgtccctgc ccattgtaca
1561 caccgcccgt cgcttctacc gattgaattg caaggtgaat tggacggatc ggccggcccg
1621 ccggtcgaga agtctcgtga atcctgtaat ttagaggaag gaaaagtcgt aacaaggttt
1681 tm
```

Figure 10.1 Sequence for *Minchinia occulta* submitted to GenBank (EF165631) with the target sequences for the probes and primers designed in the project. Green indicates target sequence for SSR66 (5'ccgcgcgatgcccagccgtat3') primer. Red letters indicate the target sequence for the SSRDb (5'gttagccttgcgcgcagccgatacg 3') probe. Yellow indicates target sequence for SSR69 (5'agg ccc aaa acc aac aaa acg tcc aca 3') primer. Grey highlight indicates target sequence for the Minch F1B (5'ctcgcg ggctcagctt3') sequence. Blue Highlight indicates the Minch R2B (5'ggcgctttgcagattccc ca3') target sequence. Bases in orange indicate the variable bases detected in the sequence generated by the Minch F1B and Minch R2B primers.

10.2 Appendix 2: Primers used to attempt to obtain a sequence from the *Haplosporidium hinei* infected pearl oyster samples.

Table 10.1 Primers used in the PCR reactions to attempt to obtain a sequence from the archived formalin fixed *Haplosporidium hinei* infected pearl oyster samples.

Primer name	Sequence 5'-3'	Reference
HAP F1	GTT CTT TCW TGA TTC TAT GMA	(Renault, T., Stokes, N.A., Chollet, B., Cochennec, N., Berthe, F., Gerard, A. and Burreson, E.M., 2000)
HAP F2	GCC RTC TAA CTA GCT	(Renault, T., Stokes, N.A., Chollet, B., Cochennec, N., Berthe, F., Gerard, A. and Burreson, E.M., 2000)
HAP R1	CTC AWK CTT CCA TCT GCT G	(Renault, T., Stokes, N.A., Chollet, B., Cochennec, N., Berthe, F., Gerard, A. and Burreson, E.M., 2000)
HAP R2	GAT GAA YAA TTG CAA TCA YCT	(Renault, T., Stokes, N.A., Chollet, B., Cochennec, N., Berthe, F., Gerard, A. and Burreson, E.M., 2000)
HAP R3	AKR HRT TCC TWGTTC AAG AYG A	(Renault, T., Stokes, N.A., Chollet, B., Cochennec, N., Berthe, F., Gerard, A. and Burreson, E.M., 2000)
18S-EUK581-F	GTG CCA GCA GCC GCG	(Carnegie, R.B., Meyer, G.R., Blackbourn, J., Cochennec-Laureau, N., Berthe, F. and Bower, S.M., 2003)
18S-EUK1134-R	TTT AAG TTT CAG CCT TGC G	(Carnegie, R.B., Meyer, G.R., Blackbourn, J., Cochennec-Laureau, N., Berthe, F. and Bower, S.M., 2003)
16 S	AAC CTG GTT GAT CCT GCC AGT	(Medlin, L., Elwood, H.J. and Sogin, M.L., 1988)
16 S	TGA TCC TTC TGC AGG TTC ACC TAC	(Medlin, L., Elwood, H.J. and Sogin, M.L., 1988)
SSUF1	AAA GAT TAA GCC ATG CAA GTC	This study
SSUF2	GCA AGT CTA WGT ATA AAC ACG	This study
SSUR1	TGT TCC TAT CAA TCA TTA CTT	This study
SSUR2	TCC AAC TAC GAG CTT TTT AAC	This study
MR1	GTC TCA ATC TTC CTT CTG CTG	This study
MF1	CGT TAG CGC CCG CTC GCG GGC	This study
MR2	GAT GAA TAA TTG CAA TCA CCT	This study
SSUBR1	TCA CCT ACG GAA ACC TTG	This study
SSUBR2	CCT TGT TAC GAC TTT TCC	This study
SSUBF1	CCT GAT CCT TTA CTT TGA	This study
SSUBF2	AAG CAT TCA CCA AGT TTG	This study
SSUF1	AAA GAT TAA GCC ATG CAA GTC	This study
SSUF2	GCA AGT CTA WGT ATA AAC ACG	This study
SSUR1	TGT TCC TAT CAA TCA TTA CTT	This study
SSUR2	TCC AAC TAC GAG CTT TTT AAC	This study